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ANTAGONISTS OF NUCLEIC ACID DERIVATIVES

I. THE LACTOBACILLUS CASEI MODEL

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A comprehensive study of the relationship between chemical structure and the rôle of pyrimidines and related substances in the biosynthesis of derivatives of nucleic acids was undertaken a number of years ago. Several preliminary accounts of this work have been given (1-5).

It was expected that such studies might serve two purposes. The biochemical pathways involved in nucleic acid synthesis might be revealed if substances could be found to block these reactions. Reversal studies might then result in the identification of previously unknown biochemical intermediates. This approach has come to be regarded with some skepticism (6) since reversals by obviously unnatural substances are not infrequently observed.[1] Nevertheless it is felt that interpretations based on the above premises are worth consideration since frequently biochemical mechanisms are suggested which can be studied by more definitive methods.

The second purpose of these studies was the discovery of new chemotherapeutic agents. The rate of nucleic acid synthesis by parasitic tissues of all kinds, whether of bacteria, rickettsiae, viruses, protozoa, or neoplasms, is apparently more rapid than that of host tissues as a consequence of the rapid rate of reproduction upon which the parasite depends for survival. An antagonist might, therefore, be expected to affect the parasite more strongly than the host on a purely differential rate basis. This type of effect probably explains the protective action of diaminopurine (7) and the structural analogues of folic acid (8) in leucemia.

A more important consideration for the elaboration of chemotherapeutic agents on this basis lies in the qualitative differences in the pathways to nucleic acid synthesis which may exist. When this work was begun, a considerable number of microorganisms were known to require preformed pyrimidines and purines for growth (9), whereas uracil, thymine, and guanine had been found to play no rôle in nucleic acid synthesis of the rat (10). Subsequently, it has been discovered that adenine (11), diaminopurine (12), and orotic acid (13) contribute to the nitrogenous fractions of nucleic acid in the rat. However, qualitative differences in the utilization of various pyrimidines and purines appear to exist between the mammal and a considerable variety of bacteria. The related requirements of protozoa, rickettsiae, and viruses are relatively unknown. *Tetrahymena geleii* utilizes guanine and uracil (14), requirements similar to those of molds (15) and bacteria (9) and unlike those of the rat. *Plasmodium gallinaceum* appears to resemble *Lactobacillus casei* in some of its requirements since a new type of antimalarial has been found among the antagonists studied in the *L. casei* screening test (16). Relatively little is known about the nitrogenous bases involved in virus multiplication, although adenine may be involved in the reproduction of the *Escherichia coli* T_2 phage (17). The multiplication of vaccinia virus *in vitro* is inhibited by a number of antimetabolites (18). Some difference between this virus and mammalian tissues in the biochemical pathways leading to nucleic acid may be indicated by the fact that virus multiplication is inhibited by diaminopurine but, in contrast to many other tissues, is relatively unaffected by the structural analogues of folic acid (18).

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[1] Elion, G. B., Falco, E. A., and Hitchings, G. H., to be published.

L. casei was chosen as a test organism for the preliminary screening of substances for activity as antagonists of nucleic acid synthesis. This choice was based on the observations of Snell and Mitchell (19) and Stokstad (20) that the folic acid requirement of this microorganism is closely related to thymine and purine synthesis, since the vitamin requirement could be replaced by these substances. This relationship allows the study of a potential antimetabolite in a variety of ways in the same microorganism.

EXPERIMENTAL

Medium—The basal medium (O) is that of Landy and Dicken (21) with omission of purines, uracil, and folic acid and supplemented or modified as follows (amounts per 100 ml. of medium): DL-alanine 20 mg., DL-glutamic acid 0.5 mg., pyridoxine hydrochloride 200 γ, *p*-aminobenzoic acid 20 mg., thiamine chloride 20 γ, glucose 1.5 gm., sodium acetate 1.0 gm., $MgSO_4 \cdot 7H_2O$ 20 mg., $FeSO_4 \cdot 7H_2O$ 4 mg., and $MnSO_4 \cdot 2H_2O$ 4 mg.

100 γ of thymine (T) or 4.6 mμgm. of folic acid (FA) per 100 ml. may be added to produce three test media (O, OT, OFA). The addition of purine (P) to the basal medium, usually 1 mg. of adenine sulfate per 100 ml., adds three modifications (P, PT, PFA), giving six test media in all (*cf.* Table I).

Growth in these media is relatively slow, but this avoids several complications which appear when growth-accelerating factors are added. For example, growth is faster when enzymatic rather than acid casein hydrolysate is used. Under these conditions uracil becomes a limiting factor for growth, but can be omitted altogether from the regular medium. This observation has been used as the basis for a study of substances with anti-uracil effects (to be published).

Inoculum—Seed cultures are grown as recommended by Snell and Strong (22), twice washed with saline, centrifuged, resuspended in saline, and 0.02 ml. added to 10 ml. of medium. The carry-over of folic acid appears to be much less than in an inoculum prepared from a culture grown in the medium (O) supplemented by 1.0 mμgm. of folic acid per ml., since use of the latter inoculum gave smaller effects of folic acid antagonists (to be published).

TABLE I

Response of L. casei to Pyrimidine Derivatives

Line No.	Substance	Concentration	Acid production in various media, ml. of 0.1 N per 10 ml.					
			O	OT	OFA	P	PT	PFA
		mg. per 10 ml.						
1	Control		0.6	0.8	6.0	1.0	6.5	8.0
2	Adenine sulfate	0.1	1.0	6.5	8.0	1.0	6.8	8.8
3	2,4-Diamino-5-formamido-6-hydroxypyrimidine	1.0	0.8	3.9	7.0	1.0	6.5	8.1
4	Thymine	0.01	0.8	0.8	5.8	6.5	6.6	10.0
5	5-Methyl-4-hydroxypyrimidine	1.0	0.9	0.9	5.5	7.0	6.5	10.2
6	Folic acid	0.46×10^{-6}	6.0	5.8	8.0	8.0	10.0	10.5
7	5-Hydroxyuracil	1.0	0.5	0.6	0.5	0.6	0.5	0.6
8	5-Aminouracil	1.0	0.3	0.5	1.0	0.4	3.3	3.2
9	5-Nitrouracil	1.0	0.3	0.6	0.5	0.5	6.0	0.8
10	5-Bromouracil	1.0	0.5	0.5	4.8	2.0	2.5	10.0
11	4-Aminofolic acid	0.5×10^{-6}	0.5	0.5	0.4	0.5	5.6	2.0
12	2,4-Diamino-5-*p*-chlorophenoxypyrimidine	0.01	0.4	0.4	0.3	0.5	4.0	0.8
13	2,4-Diamino-5,6-dimethylpyrimidine	1.0	0.2	0.2	0.2	0.3	4.2	0.5
14	2,6-Diaminopurine	1.0	0.3	0.3	0.8	1.1	6.5	7.0

O, basal medium; OT, O + 1 γ of thymine per ml.; OFA, O + 0.046 mμgm. of folic acid per ml.; P, O + 10 γ of adenine sulfate per ml.; PT, P + 1 γ of thymine per ml.; and PFA, P + 0.046 mμgm. of folic acid per ml.

Growth—The growth is expressed as ml. of 0.1 N acid produced during 68 hours of incubation at 37°. An exhaustive comparison of this procedure with other possible criteria of growth such as plate counts, turbidities, cell counts, and nitrogen determinations has been made.[2] For the purposes of this paper it is sufficient to state that lactic acid production is at least as satisfactory as any other single criterion of growth.

Results

The growth characteristics of *L. casei* under the conditions described are shown in Figs. 1 and 2. Fig. 1 shows the lactic acid production as related to the concentration of folic acid at three time intervals. It will be noted that lactic acid production is related to the folic acid concentration over the range of 0.023 to 0.575 mμgm. per ml. but that increases in folic acid concentration above this level have essentially no further effect. At 68 hours and with 0.575 mμgm. of folic acid per ml., the lactic acid found approaches the stoichiometric amount obtainable from the glucose present. At all levels of folic acid, a considerable lag period in fermentation is shown.

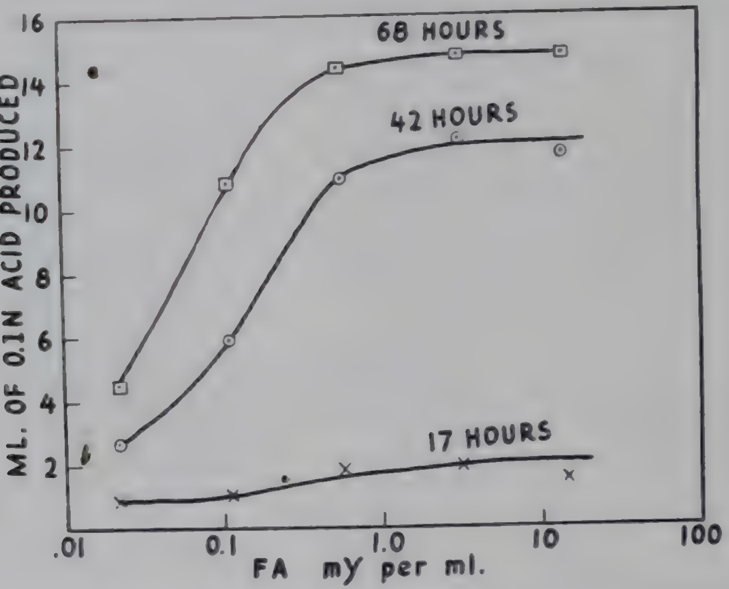

FIG. 1. Lactic acid production as affected by concentration of folic acid (FA)

For routine use, the concentration of folic acid was chosen so that the growth curve in this medium (OFA) approximates that in the medium containing thymine and adenine (PT). This is shown in Fig. 2 where the growth curves in these two media are also contrasted with that of a medium containing sufficient folic acid to produce a maximal response. Addition of further thymine does not increase the rate of lactic acid production above that observed with 10 γ per 10 ml. (23). However, the conversion of glucose to lactic acid in this medium is not limited to half maximal (23) but is simply slower than when optimal folic acid is present. This slower rate of fermentation is not a function of the number of organisms, the number of viable organisms, or the total bacterial nitrogen,[2] but seems to reflect a metabolic difference.

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[2] Lorz, D., Hitchings, G. H., and VanderWerff, H., to be published.

The use of six minor variations of the same basal medium to detect as many types of biological activity is illustrated in Table I. The basal medium (O) is supplemented with adenine (P), thymine (OT), or folic acid (OFA) and combinations of adenine and thymine (PT) and adenine and folic acid (PFA). When a substance is tested in each of these media it is often possible to determine at once the type of biological activity which it possesses. Thus the addition of adenine results in a marked increase in growth in the OT medium and an appreciable stimulation in OFA, while 2,4-diamino-5-formamido-6-hydroxypyrimidine is identified as a purine-like substance because the same pattern of biological activity is followed. The marked difference in growth in the OT medium (without adenine) and the PT medium (with adenine) illustrates the essential nature of the purine requirement when thymine is used as the nutrilite. The growth in the OFA medium shows that the purine requirement can be satisfied from endogenous reactions when folic acid is present.

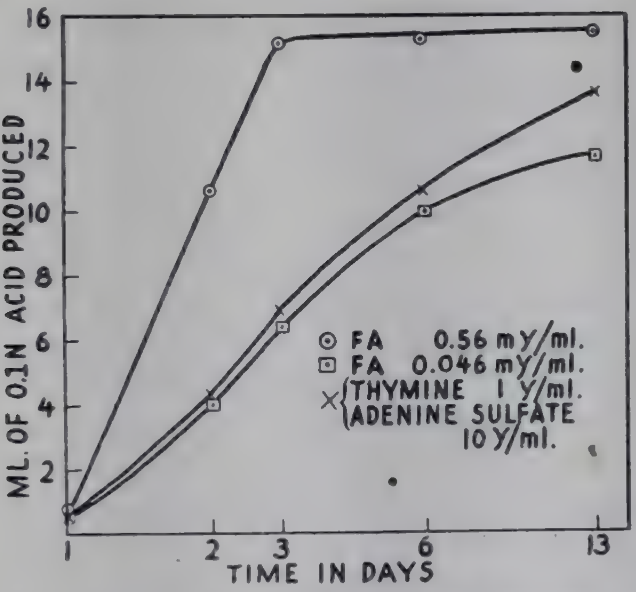

FIG. 2. Lactic acid production of *L. casei* in various media as a function of time

The effects of the addition of thymine or of a thymine-like substance (5-methyl-4-hydroxypyrimidine) can be illustrated by Lines 4 and 5 of Table I. The activity of such substances is seen primarily in the P medium, again showing that purine is essential for growth with thymine and demonstrating that 20 γ per 10 ml. of thymine do not increase the rate of growth above that obtained with 10 γ per 10 ml.

The addition of folic acid (Line 6, Table I) results in the stimulation of growth in all media, but this is especially marked in the two control media (O and P).

These media are useful also for the detection of antagonists. For the most part, separate reversal experiments are necessary to determine the exact nature of the inhibition. Thus 5-hydroxyuracil inhibits in all media and its action is reversed by neither folic acid nor thymine[3] but is reversed by uracil (5).[1] Similarly, 5-aminouracil inhibits growth with either thymine or folic acid, but its action is partially reversed by purine (the difference between OFA and PFA) and completely by either folic acid or thymine (5).[1] 5-Nitrouracil (Line 9, Table I) apparently acts as a folic acid antagonist since growth with this substance is completely blocked in both the absence (OFA) and presence (PFA) of purine, while growth with thymine (PT) is essentially unaffected. 5-Bromouracil, on the other hand, shows some tendency to simulate thymine in its effects (increase in P and PFA, decrease in OFA) but clearly blocks the utilization of thymine (decrease in PT). A structural analogue of folic acid (Line 11) resembles nitrouracil in blocking growth with folic acid but leaving growth with thymine relatively unaffected. A phenoxydiaminopyrimidine (Line 12) behaves similarly but has a somewhat greater effect on thymine growth and is somewhat less affected by the presence of purines than is the analogue of folic acid. Diaminodimethylpyrimidine (Line 13) behaves similarly. Reversal of the inhibitory action of 4-aminofolic acid, the diaminopyrimidines, and nitrouracil by folic acid can be demonstrated (5).

The action of 2,6-diaminopurine (Line 14, Table I) is seen to be directed primarily toward growth with folic acid in the absence of purine. This inhibitory activity is readily reversed by adenine (PFA). At high concentrations of the inhibitor such as that illustrated here, this reversal is specific (4, 5), although a number of purines can satisfy the normal purine requirement[4] of the organism. This inhibitor, therefore, gives the appearance of interference with endogenously produced adenine while having little influence on the utilization of exogenously supplied adenine (5).

DISCUSSION

The finding that 2,6-diaminopurine, labeled with isotopic nitrogen in the 1 and 3 positions, can be incorporated into the guanine fraction of the ribonucleic acid of the rat (12), although somewhat beclouding the mechanism of the inhibitory effects of diaminopurine, does demonstrate the participation of this substance in nucleic acid metabolism. This kind of evidence is not available for other pyrimidine derivatives, but an *a priori* case for the participation of some of these in nucleic acid metabolism can be argued on the basis of reversal experiments. The fundamental premise of this work that structural analogues of the purines and pyrimidines may be found to affect nucleic acid metabolism appears to be highly probable.

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[3] The action of this substance was erroneously stated earlier (2) to be reversible by these nutrilites.

[4] Elion, G. B., and Hitchings, G. H., to be published.

It was recognized early in this work that a substance might fulfil the criteria of competitive antagonism with a metabolite in a single microbial system without possessing the full complement of properties which are found in structural analogues of the metabolite. Thus 5-nitrouracil is not regarded as an "antifolic" in any broad sense, since its action differs from this pattern in microorganisms closely related to *L. casei* and apparently may show different effects in the same organism under different conditions (24). On the other hand, the structural analogues of folic acid show "antifolic" effects in a wide variety of biological systems (25–27).

The demonstration of the true nature of an antagonist thus depends to a considerable extent on the results of studies in a wide variety of biological systems. If a substance produces the symptoms of deprivation of the metabolite in all systems studied, it may safely be assumed that it is acting by displacement of the metabolite from cell receptors which are similar in many organisms and essential to the utilization of the metabolite. On the other hand, a substance may exhibit a consistent pattern of antagonism in a number of microbiological systems and yet fail to exhibit this antagonism in more complex organisms by reason of the intervention of pharmacological effects or metabolic changes which make it impossible to reach the concentration at which the antimetabolite effect would be expected. This explanation may apply, for example, to the lack of antifolic effects of 2,4-diamino-5,6-dimethylpyrimidine which has quite striking pharmacological properties.[5]

A somewhat intermediate classification of antimetabolites includes compounds which have similar but distinct effects in different organisms. Thus, 2,4-diamino-5-*p*-chlorophenoxypyrimidine gives a clear cut picture of folic acid antagonism in *L. casei*. It is an antimalarial approximating quinine in activity against *P. gallinaceum* (16) and this activity is *partially* reversed by folic acid *in vivo*.[6] In *T. geleii* it is inhibitory in the presence of excess folic acid and this inhibition is reversible by uracil.[7] No evidence of antifolic effects with this substance has been obtained in either mouse or chick.

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[5] Philips, F., personal communication.

[6] Greenberg, Joseph, personal communication.

[7] Kidder, G. W., personal communication.

A number of possibilities exist for the mode of action of such a substance: (1) The compound may possess several active centers which allow it to block, more or less independently, different biochemical reactions in different tissues; (2) a single reaction may be blocked in all organisms but this reaction may be of relatively greater importance to one organism than to another; (3) the dissociation of the inhibitor-cell receptor complex may vary widely from organism to organism; or (4) the reaction blocked may be one which occurs only sporadically in nature as an obligate biochemical mechanism. Whatever their mode of action, such antimetabolites provide interesting leads, for the successful elaboration of chemotherapeutic agents depends on the discovery of substances which possess considerable biological and biochemical specificity.

SUMMARY

1. A screening test is described, with *Lactobacillus casei* as the test object, for the discovery and characterization of substances capable of blocking the biosynthesis of nucleic acid and related substances.

2. The use of this screening test in the elaboration of new chemotherapeutic agents is exemplified and discussed.

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THE INFLUENCE OF CERTAIN ENDOCRINE SECRETIONS ON AMINO ACID OXIDASE*

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Deamination is one step in the metabolism of amino acids. This process is catalyzed by the D- and L-amino acid oxidases. It is known that various endocrine secretions will affect the rate of enzymatic reactions.

The present communication presents data on the interrelation of amino acid concentration in the blood and the secretions of the hypophysis, adrenal, and thyroid on the rate of activity of the liver and kidney amino acid oxidase systems. Alterations in the oxygen uptake, as measured by the Warburg manometric technique, were used as criteria for changes in the enzymatic activity of the amino acid oxidase, AAO.

EXPERIMENTAL

White male rats of the Sprague-Dawley strain, weighing 250 to 300 gm. and fed on Purina dog chow checkers, were employed. They were fasted for 18 hours before the experiment, but were permitted water. Four groups of animals were employed: normal, adrenalectomized, hypophysectomized, and thyroidectomized. Within each group a series of experiments was carried out in each of which two animals were injected intraperitoneally with a 10 per cent solution of an enzymatic casein hydrolysate,[1] while one animal was simply pierced with a needle. For convenience, the latter procedure will be called "dry needle."

The liver extracts were prepared by excising approximately 3 gm. (weighed to the nearest 0.1 mg.) of tissue from the animals (killed by decapitation) and by homogenizing the tissue with 6 ml. of phosphate buffer (pH 7.35) for 5 minutes. The buffer employed contained NaCl (0.154 M), $MgSO_4 \cdot 7H_2O$ (0.154 M), and KCl (0.154 M) and was adjusted to pH 7.35 with HCl and Na_2HPO_4. After homogenization, the minced tissue mixture was centrifuged at about 8000 R.P.M. for 10 minutes. The supernatant was decanted and 2 ml. portions were used for the enzyme activity determination. The kidney was processed in a similar manner and approximately 1.2 gm. of tissue were homogenized with 10 ml. of the same buffer for 10 minutes. According to the findings of Blanchard *et al.* (1) most of the D enzyme probably would be inactivated by the processing procedure. Dry weight determinations were made by drying the tissue at 110° for 3 hours so that corrections could be made for variation in water content of the tissue.

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* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists at Detroit, Michigan, April 18 to 22, 1949.

[1] Amigen, prepared by Mead Johnson and Company, Evansville, Indiana. We wish to express our appreciation to Dr. Warren M. Cox of that company for generously supplying us with this preparation.

It was necessary to attain a constancy of the tissue processing conditions so that the enzyme activity would vary only with the animal and its experimental environment. Accordingly, 25 and 45 minutes were allotted from the time the animals were killed until the tissue was homogenized, and from homogenization until the first readings were taken, respectively.

Since the effect of amino acid administration on AAO activity was to be determined after periods $\frac{1}{2}$ and $1\frac{1}{2}$ hours from the time of injection, animals were sacrificed at these time intervals and their tissues processed as related above.

In the Warburg manometric technique, employed for the determination of oxygen uptake, the flasks were filled as follows: main chamber, 2 ml. of buffered supernatant homogenate; side arm, experimental flask 0.2 ml. of a 0.1 M DL-alanine solution in buffer, control flask 0.2 ml. of buffer; center well, 0.2 ml. of a 10 per cent sodium hydroxide solution. After replacing the air in the flasks with oxygen, they were placed in a water bath and equilibrated for 10 minutes at 36.8°.

After tipping the substrate into the main chamber, readings were taken every 15 minutes for 1 hour while the flasks were shaken 80 times per minute. The oxygen uptake of the extracted homogenate is expressed in microliters of O_2 per gm. of wet tissue.

Experiments *in vitro* and *in vivo* have been carried out in a similar manner in order to determine the effects of insulin, adrenal cortical extract, and epinephrine on the AAO system.

Experiments in Vivo—(*a*) 0.5 I.U. of crystalline insulin (Squibb) was injected intramuscularly into the experimental animal 1 hour prior to decapitation; (*b*) 1 ml. of aqueous adrenal cortical extract (Upjohn) was injected intramuscularly hourly during a 4 hour period prior to sacrificing;[2] and (*c*) 0.3 ml. of a 1:1000 adrenalin hydrochloride solution (Parke, Davis) was injected in the same manner as (*b*).

Experiments in Vitro—0.05 I.U. of insulin, 0.1 ml. of aqueous adrenal cortical extract (Upjohn), or 0.1 ml. of 1:25,000 adrenalin hydrochloride solution (Parke, Davis) was used in each of the control and the experimental flasks which were otherwise prepared as described previously.

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[2] The control animals in this group were injected with the respective quantity of 10 per cent ethyl alcohol since the cortical extract is a 10 per cent alcoholic solution.

Control determinations were run for each group of the experiments *in vitro* and *in vivo*.

Blood glucose (2), urea (3), amino acids (4), and hematocrit determinations were carried out simultaneously on blood obtained at the time the animals were killed by decapitation.

The adrenalectomized animals were kept for 7 days on the Purina diet but given 1 per cent NaCl solution for drinking water. They then were taken off NaCl solution, given plain water for 3 days preceding the experiment, and fasted for the last 18 hours. The hypophysectomized animals were kept for 14 to 21 days on a milk, chopped meat, and bread diet before the experiment. This group had its own control group fed a similar diet. The thyroidectomized animals were maintained for at least 21 days before being subjected to experimental procedures. Only those animals that evinced a minimum decrease in basal metabolic rate of 25 per cent were used.

Sham operations (neck dissection) were performed on another group of animals in order to determine whether any effect on the AAO resulted from the operation alone. No changes were found when the results were compared with those of intact control animals.

RESULTS AND DISCUSSION

Liver Amino Acid Oxidase—From Table I it is obvious that administration of a casein hydrolysate to normal animals caused a pronounced increase in the liver AAO activity. However, neither adrenalectomized nor hypophysectomized animals similarly treated showed this increase. Therefore, the effect of the increased blood amino acid level on the liver AAO is probably mediated by the pituitary-adrenal system. The question whether the elevated blood amino acid level causes a direct or indirect stimulation of the anterior pituitary cannot as yet be answered.

The effect of the adrenal cortical secretion on the oxidase activity of the liver was also observed by experiments *in vitro* and *in vivo*. Table II illustrates that normal as well as adrenalectomized animals, given adrenal cortical extracts, showed an increased oxidase activity of the liver, whereas untreated adrenalectomized animals showed a decreased AAO activity (Table III). The experiments *in vitro* also demonstrated the accelerating effect of cortical extracts upon AAO activity of the liver (Table II).

The AAO activity of the liver of hypophysectomized animals was increased 100 per cent over that of normal animals (Table IV). This increase in activity was probably due to the absence of the pituitary growth factor and is in agreement with the concept that the growth hormone may inhibit amino acid catabolism. It has been shown by Szego and White (5) that growth hormone produces increased fatty acid metabolism and fat deposition in the liver when administered to normal fasted animals. These investigators suggested that the growth hormone may either inhibit amino acid catabolism or accelerate fat metabolism. Our observations support the former postulate, for in our experiments the hypophysectomized animals showed an increased AAO activity in the liver. Russell and Cappiello (6) recently reported that the rate of urea formation was reduced after the administration of anterior pituitary growth hormone.

TABLE I

Increase in Amino Acid Oxidase Activity after Amino Acid Injection

The values equal microliters and per cent increase in microliters of oxygen uptake of extract of tissue homogenate per gm. of tissue. Data computed from Tables III and IV.

Tissue	Time after injection	Normal		Adrenalectomized		Hypophysectomized		Thyroidectomized	
	min.	*µl.*	*per cent*	*µl.*	*per cent*	*µl.*	*per cent*	*µl.*	*per cent*
Liver	30	14	50	−5	−17			9	33
	90	15	38	1	4	−31	−38	3	11
Kidney	30	119	89	−34	−13			−43	−14
	90	18	7	107	59	66	29	−93	−27

TABLE II

Effect of Hormones on Amino Acid Oxidase of Liver In Vitro and In Vivo

Oxygen uptake of extract of tissue homogenate given in microliters per gm. of tissue. The figures in parentheses represent the number of animals. In the experiments *in vivo*, the control animals of the adrenal cortical extract group were injected with either 10 per cent ethyl alcohol (see the text, foot-note 2) or the dry needle and those of the adrenalin and insulin groups with either 1 per cent NaCl solution or the dry needle as in the experimental animals.

	Experiment	Experimental animals			Control animals
		Buffer	Alanine	Oxidase activity	Oxidase activity
Adrenal cortical	*In vitro*, normal	300	425	125 ± 18 (6)	59 ± 8 (14)
	" *vivo*, adrenalectomized	301	343	42 ± 3 (2)	21 ± 3 (2)
	" " normal	219	279	60 ± 4 (4)	27.5 ± 1.5 (2)
Epinephrine	*In vitro*, normal	232	257	25 ± 2.6 (5)	59 ± 8 (14)
	" *vivo*, "	230	253	23 ± 6 (4)	41 ± 4.5 (20)
Insulin	*In vitro*, normal	242	274	32 ± 9 (13)	59 ± 8 (14)
	" *vivo*, "	203	246	43 ± 4 (8)	57 ± 9 (4)

TABLE III

Total Respiration and Amino Acid Oxidase Activity of Experimental Animals Given 5 Ml. of Casein Hydrolysate Intraperitoneally

Oxygen uptake of extract of tissue homogenate in microliters per gm. of tissue. The figures in parentheses represent the number of animals.

Tissue	Time after injection	Normal			Adrenalectomized		
		Buffer	Alanine	Oxidase activity	Buffer	Alanine	Oxidase activity
	min.						
Liver	30	226	267	41 ± 4.5 (20)	225	249	24 ± 4.3 (19)
	90	224	279	55 ± 3.1 (21)	179	207	28 ± 4.0 (7)
Kidney	30	205	459	254 ± 36 (19)	169	400	231 ± 25 (14)
	90	207	477	270 ± 31 (28)	187	476	289 ± 20 (7)
		Hypophysectomized			Thyroidectomized		
Liver	30				219	246	27 ± 3.0 (8)
	90	200	250	50 ± 5.7 (5)	239	269	30 ± 4.3 (8)
Kidney	30				195	463	268 ± 22 (8)
	90	173	470	297 ± 30 (9)	194	451	257 ± 5 (8)

TABLE IV

Total Respiration and Amino Acid Oxidase Activity of Control Animals Given Dry Needle

Oxygen uptake of extract of tissue homogenate in microliters per gm. of tissue. The figures in parentheses represent the number of animals.

Tissue	Time after dry needle	Normal			Adrenalectomized		
		Buffer	Alanine	Oxidase activity	Buffer	Alanine	Oxidase activity
	min.						
Liver	0	211	270	59 ± 8 (10)			
	30	208	235	27 ± 3.3 (8)	197	226	29 ± 6.1 (9)
	90	235	275	40 ± 4.4 (9)	171	198	27 ± 5.1 (5)
Kidney	30	206	341	135 ± 13.5 (6)	197	462	265 ± 17 (6)
	90	220	472	252 ± 21 (13)	198	380	182 ± 14 (4)
		Hypophysectomized			Thyroidectomized		
Liver	0						
	30				205	223	18 ± 2.0 (4)
	90	204	285	81 ± 9 (5)	201	228	27 ± 3.1 (4)
Kidney	30				164	475	311 ± 18 (4)
	90	181	412	231 ± 15 (5)	201	551	350 ± 21 (4)

In thyroidectomized animals, administration of amino acids resulted in an increase in AAO activity of the liver which, however, was not as pronounced as in normal animals (Table I). According to Deane and Greep

(7), thyroidectomy leads to an atrophy of the adrenal cortex. This may explain why the increase in liver AAO activity in thyroidectomized animals was not as great as in normal animals. Thyroidectomized control animals showed a decreased liver AAO activity (Table IV), a finding which is in agreement with that of Klein (8).

Kidney Amino Acid Oxidase—Comparable data were not obtained for the kidney AAO after amino acid administration (Table I). Results for normal kidney showed an 89 per cent increase in oxidase activity after ½ hour but only a negligible increase after 1½ hours. A similar type of disagreement was also observed in the kidney AAO activity of adrenalectomized and hypophysectomized animals. Although there was no increase after ½ hour in the adrenalectomized animals, there was a 59 per cent increase in the AAO activity after 1½ hours. Again, peculiarly, a 29 per cent increase in activity occurred in the kidney of hypophysectomized animals after 1½ hours. However, in the thyroidectomized animals, the kidney AAO activity was distinctly decreased after amino acid administration. This inhibitory effect still has to be elucidated.

There appears to be a distinction between the factors influencing AAO activity of the liver and of the kidney. The AAO activity in the kidney, apparently, is not only dependent upon endocrine factors but may also be directly influenced by the amino acid level of the blood. Thus, the initial increase in AAO activity of the normal animal's kidney ½ hour after injection and the subsequent decrease after 1½ hours (Table I) may be attributed to the corresponding rise and fall of the amino acid level of the blood. This view may similarly apply to the adrenalectomized and hypophysectomized groups where the findings were reversed; *i.e.*, the greater increase in the AAO activity occurred after 1½ hours. In these instances, since the liver oxidase activity, in the absence of the activating mechanism of the pituitary-adrenal cortex system, could no longer participate normally in lowering the amino acid level, the effect of the gradual blood amino acid elevation was manifested in the kidney but not until a period of time had elapsed.

Normal and thyroidectomized animals, which were pierced with a dry needle ½ hour previous to being sacrificed, showed a decreased AAO activity in the liver and kidney compared with animals sacrificed after 1½ hours (Table IV). Since this decrease was not manifested in adrenalectomized animals similarly treated, and since it is associated with increased blood glucose levels in the normal and thyroidectomized control animals (Table VI), one may suspect that the secretion from the adrenal medulla may be responsible for the transient decrease in AAO activity. The results of experiments with epinephrine both *in vitro* and *in vivo* support this possibility (Table II). When a non-commercial epinephrine solution containing crystalline epinephrine was used, the inhibitory effect lasted for 15 to 20 minutes only. This difference in effect may have been due to the presence of antioxidants in the commercial solutions.

It is also conceivable that the above mentioned inhibition may be due to the action of insulin on the AAO. The increased blood sugar levels caused by an epinephrine reaction may result in an increased insulin secretion. Although certain investigators (9, 10) have been able to show insulin inhibition of this enzyme, it was not possible to demonstrate this effect clearly by our technique, either *in vitro* or *in vivo* (Table II).

Blood Amino Acid, Glucose, Urea, and Hematocrit (*Tables V, VI*)—AAO activity may be correlated with the level of amino acid nitrogen, urea nitrogen, and glucose in the blood. In normal animals (Table V), the amino acid nitrogen rose from 12.1 to 18.5 mg. per cent ½ hour after injection of the amino acid mixture and declined to 15.4 mg. per cent after 1½ hours, indicating rapid deamination. These variations in the blood amino acid nitrogen level can be linked with the respective changes in urea nitrogen and glucose levels of the blood (Table VI). While urea nitrogen was not greatly changed after ½ hour, it was elevated by 12.5 mg. per cent 1½ hours after the injection of amino acids. Similarly, in normal animals, there was no increase in blood glucose in ½ hour but a 10.8 mg. per cent increase occurred after 1½ hours. These results probably indicate that the increased deamination of amino acids leads to an increased formation of ammonia and, consequently, of urea and that,

TABLE V

Changes in Blood Constituents during Experimental Procedures

Hematocrit readings in per cent; amino acids in mg. per cent of amino acid N. The figures in parentheses represent the number of animals.

Blood constituents	Time after injection	Normal		Adrenalectomized	
		Control	Injected	Control	Injected
	min.				
Hematocrit	30	52.5 ± 0.6 (4)	55.4 ± 1.0 (8)	55.5 ± 1.3 (3)	60.0 ± 1.4 (5)
	90	52.4 ± 0.4 (4)	56.5 ± 1.5 (6)	55.5 ± 0.8 (3)	63.0 ± 2.0 (5)
Amino acids	30	12.1 ± 1.3 (5)	18.5 ± 1.6 (8)	12.6 ± 1.3 (7)	20.5 ± 1.3 (13)
	90	12.9 ± 1.4 (9)	15.4 ± 0.2 (12)	14.5 ± 0.6 (3)	22.3 ± 1.8 (5)
		Hypophysectomized		Thyroidectomized	
Hematocrit	30			49.4 ± 1.5 (4)	51.2 ± 0.8 (8)
	90			50.5 ± 0.9 (3)	55.0 ± 2.0 (6)
Amino acids	30			11.9 ± 0.9 (3)	21.7 ± 3.1 (6)
	90	12.5 ± 0.5 (4)	16.0 ± 1.2 (7)	12.2 ± 0.4 (4)	16.0 ± 1.6 (8)

simultaneously, increased gluconeogenesis takes place. Acceleration of these metabolic processes in normal animals had started in ½ hour and was well established in 1½ hours after the injection of the casein hydrolysate.

In the adrenalectomized animals, the increase in these transformations seemed to be slowed down or inhibited (Table V). These findings are in agreement with the generally accepted assumption that certain factors secreted by the adrenal cortex enhance protein catabolism.

The observation (Table VI) that there was a significant increase in urea formation in the adrenalectomized animals may seem surprising, since the lack of adrenal cortical secretion would lead one to believe that little or no deamination takes place. However, it must be recalled that the ability of the kidney to deaminate was not found to be as impaired as that of the liver in the adrenalectomized animals (Tables III, IV). Thus, ammonia formed in the kidneys of the animals which had been operated on may be utilized in the liver for the formation of urea. The impaired excretory capacity of the kidneys in these animals may account for retention of some urea.

TABLE VI

Changes in Blood Constituents during Experimental Procedures

The values are given in mg. per cent. The figures in parentheses represent the number of animals.

Blood constituents	Time after injection	Normal		Adrenalectomized	
		Control	Injected	Control	Injected
	min.				
Urea as urea N	30	18.9 ± 0.8 (4)	23.6 ± 1.8 (9)	40.8 ± 2.0 (3)	48.7 ± 3.1 (4)
	90	17.7 ± 0.3 (5)	30.2 ± 0.8 (8)	40.3 ± 2.6 (3)	48.6 ± 2.8 (2)
Glucose	0	68.2 ± 0.2 (3)			
	30	72.9 ± 2.1 (3)	66.6 ± 2.4 (8)	46.5 ± 2.3 (3)	36.0 ± 6.1 (5)
	90	66.0 ± 3.0 (5)	76.8 ± 2.7 (10)	47.8 ± 2.0 (3)	27.8 ± 1.1 (5)
		Hypophysectomized		Thyroidectomized	
Urea as urea N	30			28.1 ± 0.9 (3)	31.7 ± 1.8 (7)
	90	46.8 ± 2.2 (3)	59.4 ± 1.6 (6)	29.1 ± 1.5 (3)	39.3 ± 2.1 (5)
Glucose	0			58.5 ± 3.0 (3)	
	30			73.7 ± 1.0 (4)	77.6 ± 0.8 (8)
	90	45.5 ± 1.5 (2)	62.1 ± 3.6 (4)	59.2 ± 2.5 (4)	46.6 ± 3.4 (8)

Administration of amino acids to adrenalectomized animals was accompanied by a decrease in blood glucose, whereas the normal animals showed a hyperglycemia after an initial decrease (Table VI).

In hypophysectomized animals, the amino acid nitrogen level (16.0 mg. per cent) 1½ hours after amino acid administration was the same as that found in normal animals similarly treated (Table V). The control animals in the hypophysectomized group had an amino acid nitrogen level comparable to the normal controls (12.5 mg. per cent). The ability of the hypophysectomized animals to deaminate injected amino acids at a rate comparable to that observed in the normal animal may be due to the absence of the pituitary growth factor, as noted previously. Furthermore, the absence of this factor may explain the apparently accelerated protein catabolism, as shown by the finding that blood urea nitrogen was increased from 46.8 to 59.4 mg. per cent and glucose from 45.5 to 62.1 mg. per cent during this period (Table VI).

By the same criteria, thyroidectomized animals manifest the ability, although somewhat retarded, to metabolize amino acids. From Table V, it may be observed that the lowering of the amino acid nitrogen level was not quite as prompt or as marked but eventually, after injection of amino acids, the amino acid nitrogen level decreased from 21.7 mg. per cent in ½ hour to 16.0 mg. per cent in 1½ hours. Similarly, the urea nitrogen levels, although increased, do not quite attain the increase that the normal animals showed in ½ and 1½ hours. This finding supplements the previously mentioned phenomenon of decreased liver AAO activity in thyroidectomized animals. The blood glucose levels in these animals increased after ½ hour (3.9 mg. per cent) but decreased greatly (13 mg. per cent) 1½ hours after injection.

The higher concentration of the blood urea in thyroidectomized control rats compared with normal control animals (Table VI) is probably due to the increased renal AAO activity in these animals (Table IV). Apparently, thyroidectomy produces an elevated *resultant* catabolism of amino acids. Such a postulation is in agreement with the findings of Persike (11) and of Rupp, Paschkis, and Cantarow (12).

It can be noted from Table V that the hematocrit was increased 6 to 8 per cent in the injected normal animals and 8 to 12 per cent in the adrenalectomized animals.

SUMMARY

1. Data have been presented relating the blood amino acid level and the secretions of the hypophysis, adrenal, and thyroid to the activity of amino acid oxidase in the liver and the kidney of rats.

2. Administration of a casein hydrolysate to normal rats produces an increase in the amino acid oxidase activity of the liver and kidney in these animals.

3. Administration of a casein hydrolysate to adrenalectomized or hypo-

physectomized animals does not produce this increase in the amino acid oxidase activity of liver.

4. Adrenal cortical extract accelerates the activity of this enzyme in the liver *in vitro* and *in vivo*.

5. The pituitary-adrenal cortex system mediates the stimulus for the acceleration of amino acid oxidase activity of the liver observed after amino acid administration but the nature of the stimulus remains to be determined.

6. Livers of hypophysectomized animals show increased amino acid oxidase activity which may be due to the absence of the growth factor of the pituitary.

7. Thyroidectomized animals show a decreased amino acid oxidase activity of the liver but an increased activity of the kidney. Administration of amino acids stimulates the liver oxidase.

8. Epinephrine inhibits amino acid oxidase activity of the liver. The effect of insulin is doubtful under our experimental conditions.

9. The amino acid oxidase activity of the liver and the kidney may respond similarly to certain endocrine stimuli. However, it appears that the blood amino acid level may influence the kidney oxidase activity directly but not that of the liver.

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THE FATE OF URIC ACID IN MAN*

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The traditional assumption that uric acid represents the final end-product of nucleic acid purine metabolism in man has received rather tenuous support from balance studies (1, 2) and from the failure to detect uricase in human tissues (3, 4). An opportunity to study directly the fate of uric acid in man was afforded by the availability of isotopically labeled uric acid (5) and this has now been done with uric acid administered both orally and intravenously.

EXPERIMENTAL

Isolation of Uric Acid from Urine—It was found that uric acid in human urine could be quantitatively adsorbed on charcoal (Darco) and that it could be eluted with hot 0.1 N sodium hydroxide solution. In a typical experiment, 430 ml. of filtered human urine were mixed with about 4 gm. of charcoal and placed overnight in the refrigerator. The charcoal was collected by filtration and was eluted by boiling with 30 ml. and then with 10 ml. of 0.1 N NaOH. The filtrate was chilled and was then acidified to about pH 4 with glacial acetic acid, whereupon 100 to 150 mg. of crude uric acid were precipitated. The crude uric acid was purified by dissolving it in a minimum volume of boiling water to which a few drops of 1 N NaOH had been added. The resulting solution was decolorized with a small amount of charcoal, then neutralized with acetic acid and concentrated at the boiling point to a small volume. The crystals obtained after chilling were dissolved in a small volume of 0.1 N NaOH, decolorized again if necessary, and reprecipitated with glacial acetic acid. The last purification step was repeated. The identity and homogeneity of the product were confirmed by counter-current distribution (6). In the experiment cited, 38 mg. of uric acid were obtained. Analytical values varied from 32.9 to 33.9 per cent N, compared with a theoretical N content of 33.4 per cent.

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Presented in part before the meeting of the American Society of Biological Chemists at Detroit, May 1, 1949.

Isolation of Urea—Urea was isolated from urine as the dixanthydrol derivative (7). The extent to which the urea isolated by this procedure was contaminated with uric acid was determined. In three experiments the N^{15} content[1] of urea ranged from 0.006 to 0.008 atom per cent excess when isolated from urines to which isotopic uric acid had been added to bring the levels of N^{15} in the total uric acid from 0.55 to 0.76 atom per cent excess N^{15}. Since the abundance of N^{15} in urea from normal urine with respect to the reference standard in use is, as indicated below, 0.005 atom per cent excess, the average contamination of the urea by uric acid was thus 0.003 atom per cent, which represents not over 0.5 per cent of uric acid nitrogen in the isolated urea samples. The N^{15} level in urea isolated from a sample of urine containing uric acid with an N^{15} content of only 0.07 atom per cent excess was undetectable.

Isolation of Ammonia—Urine samples were made alkaline and aerated into standard dilute hydrochloric acid.

Determination of Abundance of N^{15} in Uric acid, Ammonia, and Urea in Normal Urine—The abundance of N^{15} in the urea isolated from the urine of three normal individuals was compared with the tank nitrogen which was in use as the reference standard. A total of fifteen independent analyses, each in triplicate, on these samples of urea was made and found to range from 0.0034 to 0.0050 with a mean of 0.0044 and a standard deviation of ±0.0005 atom per cent excess N^{15}. On another day a series of eight determinations gave a mean of 0.0048 atom per cent excess. The mean for both series was 0.0045 atom per cent. A number of determinations of the N^{15} in the total nitrogen, the ammonia, and the uric acid from normal urines were of about the same magnitude. Consequently, 0.005 atom per cent excess N^{15} has been subtracted from each value listed here to compensate for the difference in abundance of N^{15} in the reference standard and in biological nitrogen. The increased abundance of N^{15} (above that of the nitrogen of air) reported previously (8) is in agreement with the more precisely determined figure of 0.005 atom per cent given here.

The standard deviation from the mean for the abundance of N^{15} in the urea from urine was ±0.0005. However, these determinations were all made at the same time and a wider variation may be expected among samples run under varying conditions. Seventeen determinations made over a period of 11 months on a sample of guanine, containing 0.143 atom per cent excess N^{15}, showed a standard deviation of 0.0013 atom per cent excess. This figure represents the precision obtained from day to day and it was decided that only differences about 2 times this standard deviation, or 0.002 atom per cent, were to be considered significant.

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[1] Consolidated Nier mass spectrometer, model 21-201.

Determination of Uric Acid in Normal Human Urine by Means of Isotope Dilution (*9*)—Three separate random pools of urine were collected from several normal male adults. Precisely weighed samples of about 40 mg. of uric acid containing 2.28 atom per cent excess N^{15} were dissolved in a few ml. of 0.1 N NaOH and were added to 450 ml. specimens of the filtered urines. Charcoal was added after thorough mixing and uric acid was isolated and purified in the usual manner. A similar analysis was carried out on urine from the individual who was the subject of the experiments reported here. Uric acid determinations by the Archibald method (10) were carried out on the urine samples to which no isotopic uric acid had been added. The results listed in Table I indicate that the colorimetric method yields uric acid values about 16 per cent (average of Specimens 1, 2, and 4) higher than the true uric acid level in the urines. Accordingly, all of the quantitative uric acid values recorded here have been obtained by dividing the colorimetric value by the factor 1.16 to correct for the difference in the results obtained by the colorimetric (Archibald) determination and the isotope dilution method of determination.

TABLE I

Comparison of Isotope Dilution and Colorimetric Methods for Determination of Uric Acid in Human Urine

Urine Specimen No. (450 ml. each)	Isotopic uric acid added	N^{15} content of added uric acid	N^{15} content of isolated uric acid	Uric acid in total sample		
				By isotope Dilution	By colorimetric determination	Factor
	mg.	*atom per cent excess N^{15}*	*atom per cent excess N^{15}*	*mg.*	*mg.*	
1	39.9	2.28	0.524	134	158	1.18
2	39.7	2.28	0.491	145	163	1.12
3	39.9	2.28	0.386	196	253	1.29
4	40.1	2.28	0.299	265	309	1.17

Oral Administration of Uric Acid—An 88 kilo normal adult male, who followed a reasonably low purine diet, ingested 154 mg. of uric acid containing 16.0 atom per cent excess N^{15} (5) preceding a light meal. Complete 6 hour urine collections were made for the first 3 days and 12 hour collections for the next 2 days. The volumes were recorded and the urines were preserved under toluene and kept in the refrigerator. Since it was found that toluene, when present in large amounts, interfered with the adsorption of uric acid by charcoal, the toluene layer was removed prior to the determination. Samples were removed and total N and N^{15} were determined after Kjeldahl digestion. Uric acid was determined colorimetrically (10); urea was determined by the urease method and ammonia by aeration.

TABLE II

Urinary Excretion of Nitrogenous Components

Day No.	Period No.	Volume	Total nitrogen		Urea nitrogen		Uric acid nitrogen		Ammonia nitrogen	
After oral administration of uric acid (8.22 mg. N^{15})										
		ml.	*atom per cent excess N^{15}*	*mg. N^{15}*	*atom per cent excess N^{15}*	*mg. N^{15}*	*atom per cent excess N^{15}*	*mg. N^{15}*	*atom per cent excess N^{15}*	*mg. N^{15}*
1	1	200	0.005	0.19	0.000	0.00	0.163	0.29	−0.001	0.000
	2	310	0.031	1.36	0.020	0.68	0.260	0.55	0.010	0.009
	3	425	0.020	0.93	0.019	0.69	0.205	0.28	0.013	0.022
	4	380	0.019	0.78	0.019	0.63	0.162	0.25	0.009	0.015
2	5	379	0.016	0.43	0.015	0.43	0.140	0.17	0.006	0.008
	6	225	0.012	0.42	0.013	0.35	0.112	0.21	0.010	0.011
	7	460	0.009	0.32	0.006	0.19	0.091	0.09	0.007	0.010
	8	365	0.011	0.40	0.009	0.26	0.082	0.14	0.008	0.012
3	9	1055	0.008	0.56	0.007	0.40	0.060	0.15	0.007	0.019
	10	650	0.007	0.52	0.005	0.26	0.038	0.11	0.003	0.001
Total, *mg*				5.91		3.89		2.24		0.107
" *% of dose*				72		47		27		1.3
After intravenous administration of uric acid (8.33 mg. N^{15})										
1	1	1050	0.037	2.87	−0.003		1.86	3.15	0.015	
	2	600	0.023	1.73	0.000		1.19	1.45	0.001	
2	3	800	0.014	1.09	0.002		0.801	1.27	0.001	
	4	800	0.010	0.72	0.003		0.520	0.65	0.001	
3	5	1550	0.003	0.42	0.003		0.290	0.83	0.001	
4	6	1550	0.002	0.30	0.001		0.151	0.39	0.001	
5	7	1930	0.002	0.30	0.001		0.073	0.21	0.000	
Total (3 days), *mg*				6.83				7.35		
" (3 "), *% of dose*				82				88		
" (5 "), *mg*				7.43				7.95		
" (5 "), *% of dose*				89				95		

Intravenous Administration of Uric Acid—6 months after the first experiment, the same subject received a total of 156 mg. of labeled uric acid (16.0 atom per cent excess N^{15}) as a 0.5 per cent sterile solution in 0.7 per cent Li_2CO_3 in three intravenous injections of equal volume spaced 30 minutes apart. Total 12 hour urine specimens were collected for the first 2 days and complete 24 hour specimens thereafter for 3 days. The isolations and determinations were carried out as before.

Results

The values from both experiments are listed in Table II, and have been corrected for natural N^{15} abundance above that of the reference standard.

Of the total of 8.22 mg. of N^{15} orally ingested in the form of uric acid, 5.91 mg. or 72 per cent of the administered dose had been excreted within 3 days. In terms of the various forms excreted, 2.24 mg. of N^{15} or only 27 per cent of the dose was recovered as unchanged uric acid, whereas 3.89 mg. of N^{15} or 47 per cent of the ingested uric acid had been degraded to urea. The N^{15} of the urinary ammonia amounted to but a small fraction, less than 2 per cent of the ingested uric acid N^{15}. The course of excretion of the N^{15} is shown graphically in Fig. 1, and the total recoveries of N^{15} as uric acid and urea are presented in Fig. 2.

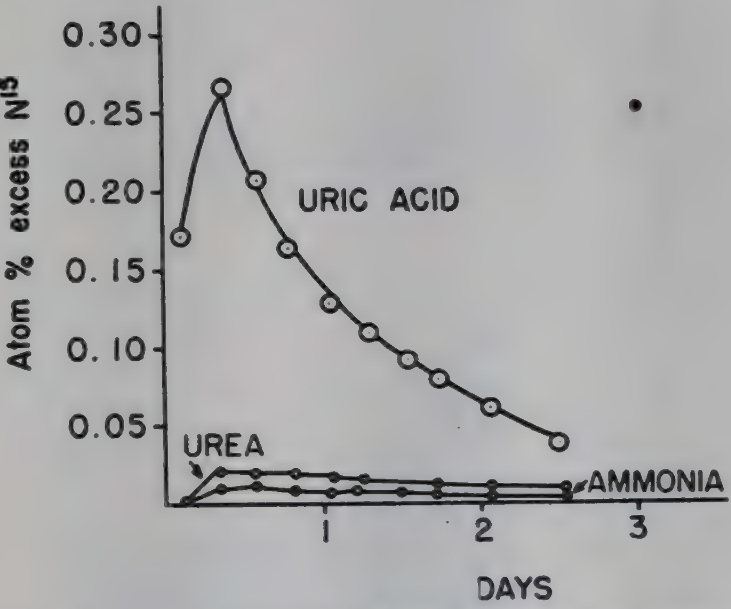

FIG. 1. Course of urinary excretion of N^{15} following oral administration of isotopic uric acid.

In the intravenous experiment, the total recovery from 8.33 mg. of injected uric acid N^{15} was 7.35 mg. of N^{15} as uric acid (88 per cent) after 3 days and 7.95 mg. of N^{15} (95 per cent) after 5 days. The course of the excretion of the N^{15} is plotted in Fig. 3 and the total recovery in Fig. 4.

The question arises as to whether there was any conversion of uric acid to urea following the intravenous injection of uric acid. This question involves a consideration of the errors involved in the calculation of our values. The value for the mean difference between the N^{15} content of biological nitrogen and of the reference standard was 0.0045, S.D. ±0.0005, and a small variation of this mean value can lead to a difference in the correction factor of 0.001 atom per cent. In the case of the total nitrogen and the urea nitrogen many of the N^{15} values were very small. It was calculated that a constant difference of 0.001 in the correction factor applied amounts to a 5 per cent change in the total N^{15} excretion for these components. Values for these components were therefore in error by ±2.5 per cent. In addition, the variations possible in each determination of N^{15} introduce another but smaller error which, calculated from the standard deviation of ±0.0013, amounts to from ±0.7 to ±2.3 per cent. In the case of the high values for the uric acid nitrogen these sources of error are insignificant, amounting to 0.3 and 0.1 per cent respectively in the two experiments.

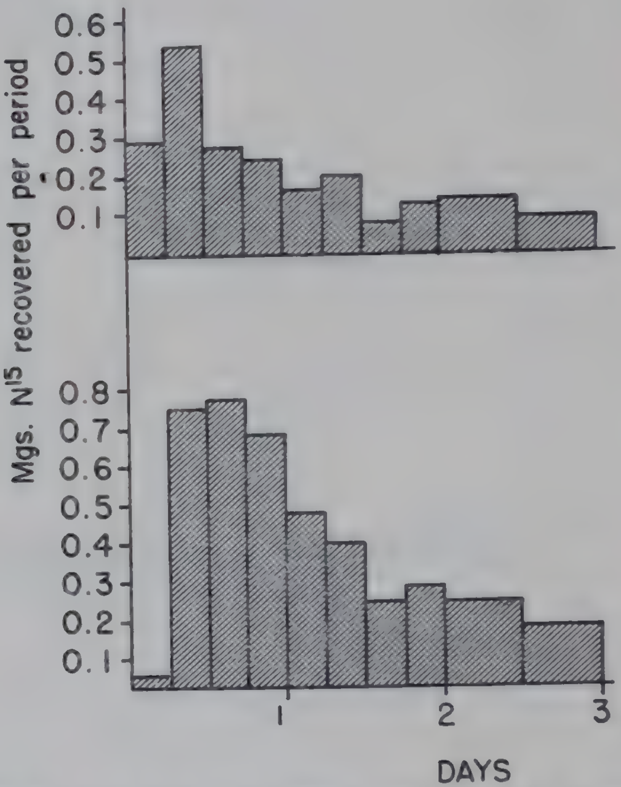

FIG. 2. Recovery of uric acid N^{15} (upper) and urea N^{15} (lower) following oral administration of isotopic uric acid.

In the intravenous experiment net values for the N^{15} in the urea (Table II) may be calculated to be 0.51 mg. or 6 ± 4 per cent of the administered dose in the first 3 days and 0.77 mg. or 9 ± 5 per cent in 5 days. In view of the magnitude of the errors involved in the very small N^{15} values from which these were calculated, we do not feel that our results afford satisfactory evidence that there is a significant conversion of the intravenously administered uric acid to urea.

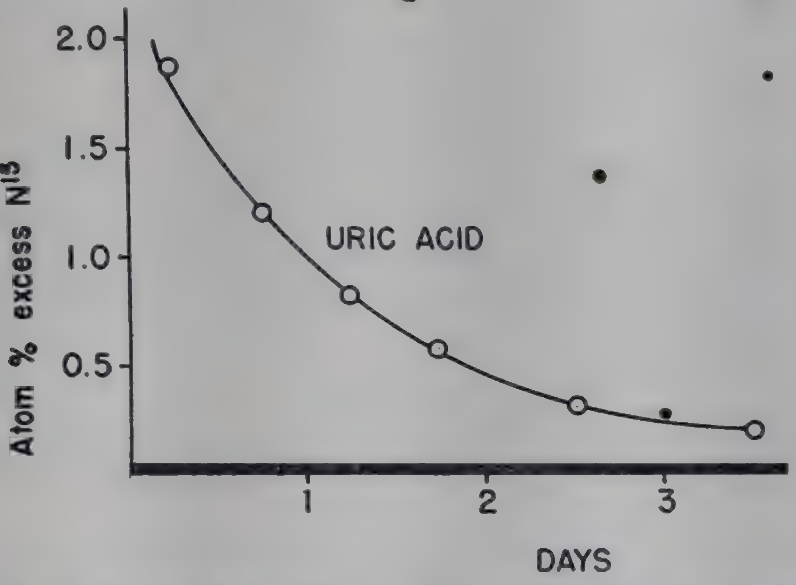

FIG. 3. Course of urinary excretion of N^{15} following intravenous administration of isotopic uric acid.

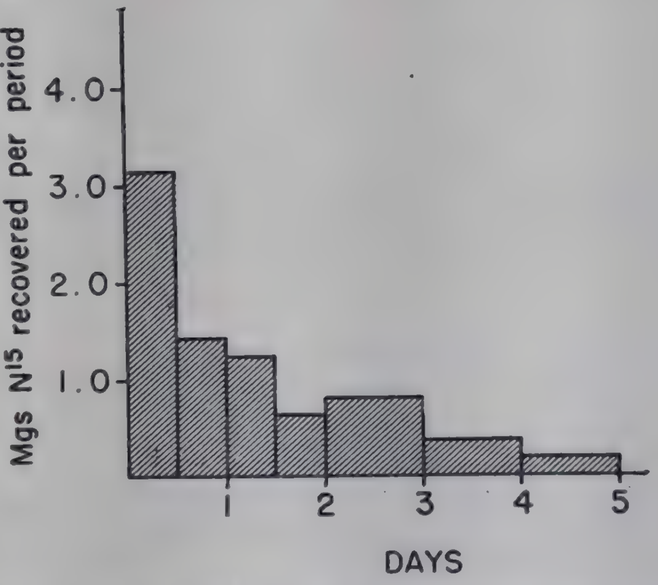

FIG. 4. Recovery of uric acid N^{15} following intravenous administration of isotopic uric acid.

Course of Excretion of Intravenously Administered Uric Acid—The simple assumption that the rate of excretion of intravenously injected uric acid is proportional to the amount of this substance present at any time leads to either of two equations (11). The first is

$$\frac{dx}{dt} = k(A - X) \tag{1}$$

in which A is equal to the amount of uric acid initially present expressed as mg. of N^{15} excess, and X is equal to the amount of uric acid expressed as mg. of N^{15} excess excreted at time t.

The second type of equation is

$$-\frac{dN}{dt} = KN \tag{2}$$

in which N is the concentration present at any time, t, in the uric acid, expressed as N^{15} atom per cent excess, and K is similarly a proportionality constant. The use of this equation does not necessitate a knowledge of the absolute amounts of N^{15} excreted but merely of its concentration in the uric acid nitrogen.

TABLE III

Conformance of Excretion of Uric Acid with Equation $k = 1/t \ln (A/(A-X))$

t	X	$A - X$	$\frac{A}{A-X}$	k
days	*mg. N^{15} excess*	*mg. N^{15} excess*		*day^{-1}*
0	0	8.33		
0.5	3.15	5.18	1.61	0.95
1.0	4.60	3.73	2.23	0.80
1.5	5.87	2.46	3.38	0.82
2.0	6.52	1.81	4.60	0.77

In order to see whether our data conform with the assumption made above, Equations 1 and 2 may be transformed into their usual integral forms.

$$k = \frac{1}{t} \ln \frac{A}{A - X} \tag{1'}$$

$$-\ln N + C = Kt \tag{2'}$$

Table III shows the results of substituting our data in Equation 1′. It may be seen that k has a fairly constant value for practically the entire course of excretion. The value of k for $t = 0.5$ day is somewhat high; only the last three values for k were averaged and yielded a value of $k = 0.80$ day^{-1} and a value for the half life, $t_{0.5}$, for the injected uric acid of 0.87.

Equation 2′ was also employed. It is apparent that the concentration of N^{15} in the uric acid of the urine is the same as that in the uric acid of the body. Hence, determination of the rate of change of concentration of N^{15} in the urinary uric acid represents the rate of change in the body. When natural logarithms of the values for the atom per cent excess N^{15}

of the uric acid for the various periods were plotted against the mid-points of these periods, a straight line was obtained (Fig. 5). This result substantiated the assumption inherent in Equation 2′. The value of K was 0.83 day^{-1} and the value for the half life ($t_{0.5}$) was 0.83 day.

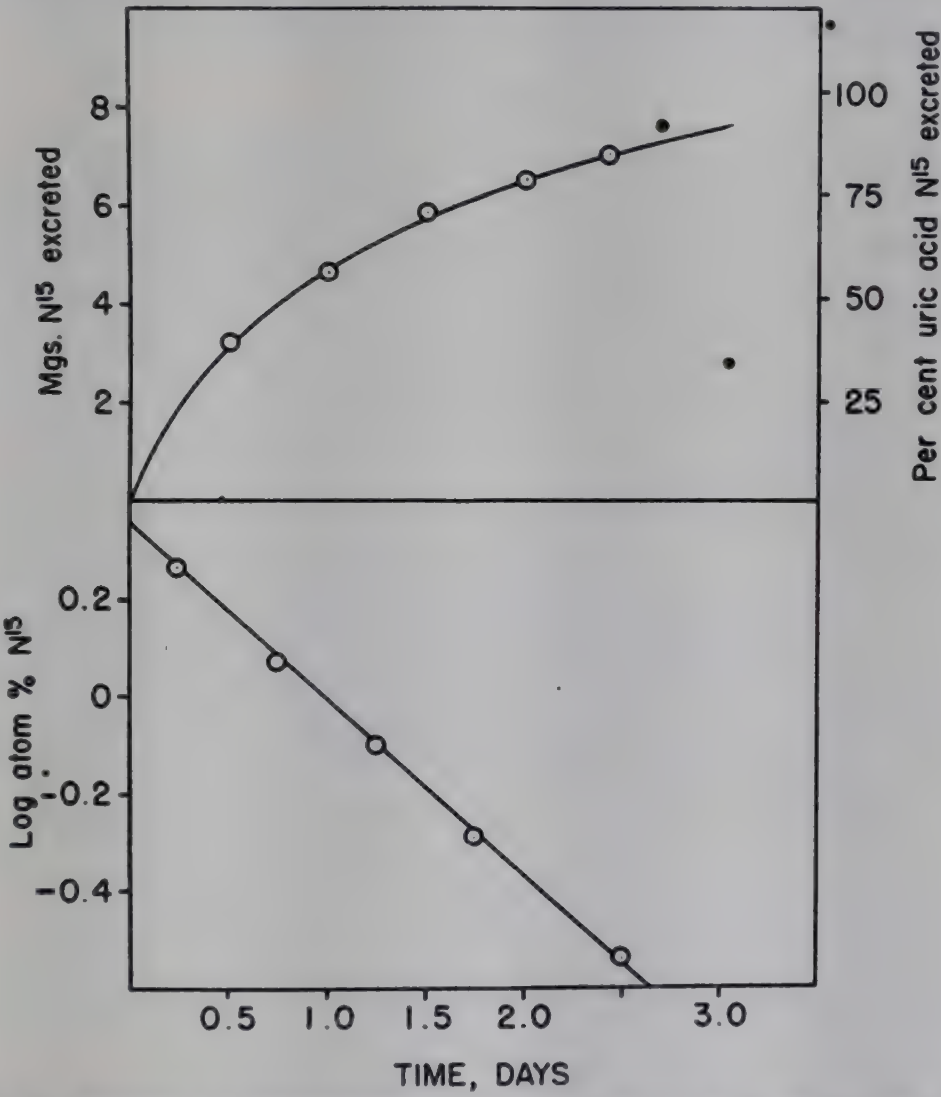

FIG. 5. Excretion of intravenously administered isotopic uric acid; upper curve, cumulative totals (*cf.* Equation 1′); lower curve, log atom per cent N^{15} (*cf.* Equation 2′).

The value for the concentration of N^{15} at zero time, extrapolated from the plot of Fig. 5, was 2.28 atom per cent N^{15}. Since the concentration in the 156 mg. of uric acid which was injected was 16.0 atom per cent N^{15}, the body pool at zero time after injection was $156 \times 16.0/2.28 = 1090$ mg. of uric acid. When this was corrected for the injected uric acid, a value of 944 mg. was obtained for the total available uric acid in the individual.

A value of 4.6 mg. per cent of uric acid was found in the blood of the experimental subject immediately before the injection. From this and the total pool of available uric acid a "uric acid space" of about 20 liters for this individual may be calculated. Thus the available uric acid was present in a volume in accordance with the usual value for the extracellular space in an individual of this weight, 88 kilos.

DISCUSSION

In the case of the orally administered uric acid, the appreciable amount of N^{15} excreted in the urea of the urine indicated unequivocally that extensive degradation of the uric acid had resulted. However, in the case of the intravenously administered uric acid, essentially all of the administered isotopic nitrogen was recovered as urinary uric acid, and the excretion of N^{15} in the urea fractions was insignificant. These results support the contention that uric acid represents the final stage of the oxidation of purines in man and are in agreement with the reported absence of uricase in human tissues (3, 4).

In view of the lack of degradation of the intravenously administered uric acid, it must be concluded that the degradation of the orally administered uric acid is associated with its passage through the gut. It is most probable that intestinal bacteria are responsible (12), the products being absorbed from the gut and excreted in the urine. If this is the case, the variability in the recoveries of administered uric acid (1), purines (13), and nucleic acid derivatives (14) reported from time to time may be due to variations in the bacterial population in the individuals involved.

It is of interest to recall that urea did not arise from orally ingested uric acid in the rat (15), although there was very extensive conversion to allantoin. It may be that the intestinal flora in the two species are different, or that, in both species, the bacteria converted some uric acid to allantoin only. In this case the rat tissues would not degrade that allantoin further, while the human may be able to do so.

The possibility that ingested uric acid is converted in the gastrointestinal tract to some derivative, for instance to the riboside (16), must be admitted. Such a derivative might be oxidized to urea, whereas uric acid is not.

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SUMMARY

It has been shown in man that orally administered uric acid is extensively degraded to urea, whereas intravenously administered uric acid is excreted essentially unchanged.

The pool of available uric acid in man has been shown to be somewhat less than 1 gm. and the half life, $t_{0.5}$, of this uric acid is less than 1 day (0.85 day).

A method for the isolation of uric acid and a comparison of the uric acid content of normal human urine, as determined colorimetrically and by the isotope dilution technique, have been presented.

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STUDIES ON THE UTILIZATION OF ACETIC ACID FOR THE BIOLOGICAL SYNTHESIS OF CHOLESTEROL*†

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Previous work (1, 2) has demonstrated that acetic acid is an important source of carbon and hydrogen in the total synthesis of steroids by the animal body, and that this compound is superior in this respect to various other organic molecules. Experiments with deuterioacetate led to the conclusion that 2-carbon compounds participate in the synthesis of both the nucleus and of the side chain of cholesterol and that, of the carbon and hydrogen atoms in the steroid molecule, at least half is derived from those of acetate. The present investigation was undertaken in order to examine the rôle of acetic acid in greater detail. To this end cholesterol synthesis has been studied with the aid of acetic acid labeled in either the carboxyl or methyl group, or acetic acid labeled by C^{13} as well as C^{14}. In this way the relative utilization of the 2 carbon atoms of acetic acid for the synthesis of cholesterol as a whole, as well as for the aliphatic and cyclic moieties of the molecule, could be determined. In a few cases the source of individual carbon atoms of cholesterol has been identified. By degradation of isotopic cholesterol the origin of the isopropyl group in the sterol side chain, of the angular methyl groups, and of 2 carbon atoms in the ring system has been established.

The labeled cholesterol used in the present experiments was obtained by synthesis *in vitro* in surviving rat liver (3). Under these conditions the isotopic substrate is utilized with greater economy than in intact animals. In order to secure the relatively large quantities of steroid which were necessary for degradation studies, the biosynthesis was carried out with acetate labeled by C^{14} of high specific activity. The resulting cholesterol could then be diluted considerably by normal material.

DISCUSSION

Although the earlier work with deuterioacetate and with acetic acid labeled in the carboxyl group has shown qualitatively that both carbon atoms

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of this compound participate in cholesterol formation, it was unknown whether they were utilized equally in the synthetic process. Since cholesterol has an odd number of carbon atoms, it is clear that, unless other precursors exist, the carboxyl and methyl carbon atoms of acetic acid cannot appear in the sterol molecule in equal numbers. Also, the presence in cholesterol of branched structures, such as the isopropyl group of the aliphatic chain, suggests that decarboxylations may occur at an intermediate stage of the synthesis. To what extent the 2 carbon atoms of acetic acid are utilized may be determined with the aid of acetic acid in which 1 of the carbon atoms is labeled by C^{13} and the other by C^{14}. The ratio of the 2 carbon isotopes in cholesterol compared to the isotope ratio in the precursor will indicate the relative proportion of methyl and carboxyl carbon atoms of acetic acid in the synthetic product. The data in Table I indicate that the ratio of methyl carbon to carboxyl carbon in cholesterol is significantly greater than 1 and hence that methyl carbons predominate. If it is assumed that all carbon atoms of the sterol are furnished by acetate, then the observed ratio of 1.27, the average value of Experiments I and II, indicates that of the total of 27 carbon atoms in the sterol molecule 15 are derived from methyl groups and 12 from carboxyl groups, respectively, of acetic acid. This deduction will be valid only if acetic acid is the sole carbon source for cholesterol and if a given carbon atom of cholesterol is derived from either a carboxyl or methyl carbon atom of acetic acid but

TABLE I

Utilization of Methyl and Carboxyl Carbon Atoms of Acetic Acid for Cholesterol Synthesis

Precursor	Cholesterol				$\frac{\text{Methyl carbon}}{\text{Carboxyl carbon}}$
	C^{13}	C^{14}	Relative isotope concentration* due to		
			Methyl carbon	Carboxyl carbon	
	atom per cent excess	*counts per min.*			
Experiment I. $C^{13}H_3C^{14}OOH$· C^{13}, 9.26 atom % excess; C^{14}, 26,100 c.p.m.	0.200	426	2.16	1.64	1.32
Experiment II. $C^{14}H_3C^{13}OOH$· C^{13}, 9.9 atom % excess; C^{14}, 103,000 c.p.m.	0.307	3900	3.78	3.09	1.22

* Relative isotope concentration =

$$\frac{\text{atom \% excess } C^{13} \text{ or counts per min. } C^{14} \text{ in cholesterol}}{\text{atom \% excess } C^{13} \text{ or counts per min. } C^{14} \text{ in precursor}}$$

not from both. Furthermore the carbon atoms of acetic acid which serve as the immediate source should have the same isotope concentration regardless of their subsequent location in cholesterol. As is shown later, the isotope concentrations observed for individual carbon atoms of cholesterol are on the whole consistent with these assumptions.

The calculation of the methyl carbon to carboxyl carbon ratio required four isotope analyses. Since the error of each determination is 5 per cent, the probable error of this ratio is about 10 per cent. Nevertheless, the close agreement of the data from two different experiments suggests that the ratio of 1.27 has real significance.[1] An independent check of the accuracy of the analytical values is provided by the isotope ratios in the saturated fatty acids which were isolated from the same experiments. In accord with previous findings, which indicated that the higher fatty acids are formed by multiple condensation of 2-carbon units (4), the ratio of methyl carbon to carboxyl carbon in the saturated fatty acids in two experiments was found to be 0.98 and 1.08.

The value of 1.27 for the ratio of methyl carbon to carboxyl carbon shows that in the course of cholesterol formation from acetate decarboxylations take place. There is no evidence to indicate at which stage of the condensation process carboxyl groups are removed. However, it may be mentioned here that, as shown later in this report, the isopropyl portion of the cholesterol side chain is composed of two methyl groups and one carboxyl group of acetic acid, and hence may result from the condensation of 2 molecules of acetate to a 4-carbon compound and its subsequent decarboxylation to a 3-carbon unit.

The participation of acetic acid in the synthesis of both the isooctyl and cyclic moieties of cholesterol had been established with the aid of deuterium as a tracer (1). The quantitative significance of the results obtained on thermolysis of deuteriocholesteryl chloride was open to question, since the drastic conditions necessary for the splitting of the cholesterol molecule could have resulted in intramolecular hydrogen shifts. We have therefore repeated the degradation with cholesterol derived from C^{14}-acetate and have determined the C^{14} content of the isooctane-isooctene mixture and of the hydrocarbon $C_{19}H_{30}$, fractions which represent the side chain and the nucleus of cholesterol. The results obtained (Table II) with cholesterol synthesized from carbon-labeled acetic acid confirm our earlier conclusion that the carbon atoms of acetic acid are utilized in the formation of both the aliphatic and the cyclic portion of cholesterol. The isotope concentration of the nucleus compared to that of the side chain was found to depend on the position of the label in the precursor. In the case of cholesterol derived from deuterioacetate, the lower isotope concentration in the nucleus may merely be due to the fact that hydrogen bound to methyl groups accounts for more than half of all the hydrogen atoms in the isooctyl side chain but only for 20 per cent of all the hydrogen atoms in the ring structure. It may be assumed that, during the condensation process, the deuterium in those methyl groups of acetic acid which still appear as methyl groups in cholesterol is less likely to be lost or replaced by ordinary hydrogen than the deuterium of methyl groups which become part of the ring structure. The isotope concentrations in the two portions of the sterol did not differ significantly when acetic acid labeled by C^{14} in the methyl group was the precursor. On the basis of these data methyl groups of acetic acid appear to supply approximately the same fraction of the carbon atoms in the side chain and in the nucleus of cholesterol. On the other hand, in cholesterol formed from $CH_3C^{14}OOH$, the radioactivity was significantly higher in the nucleus, indicating that the carboxyl group of acetic acid contributes a greater share of the ring carbon atoms than of those in the side chain. The observation that the isotope distribution in the two moieties of cholesterol depends on the position of the label in the acetic acid used could not be explained readily if the 2 carbon atoms of acetic acid participated equally in cholesterol synthesis. However, it is consistent with the above finding that a greater number of methyl than carboxyl carbon atoms of acetic acid is employed in steroid synthesis. From the data in Tables I and II, it follows that the ratio of methyl carbon to carboxyl carbon, which is 1.27 for the total cholesterol molecule, must be greater than this value for the side chain and smaller than 1.27 in the cholesterol nucleus. The ratios which are in nearest agreement with the experimental data are 5:3 or 1.67 for the isooctyl side chain and 10:9 or 1.1 for the cholesterol nucleus. On the basis of this distribution of

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[1] If the ratio methyl carbon to carboxyl carbon were 16:11 or 14:13, the isotope ratios should be 1.45 and 1.08 respectively.

TABLE II

Incorporation of Labeled Acetic Acid into Cholesterol and Degradation Products

Precursor	Atom per cent excess D or counts per min. C^{14} in			
	Cholesterol	Nucleus ($C_{19}H_{30}$) (*A*)	Isooctyl side chain (*B*)	$\frac{(A)}{(B)}$
Deuterioacetic acid*	0.21	0.18	0.26	0.69
Acetic acid-2-C^{14}	71	76	74	1.03
" "		400	424	0.94
" acid-1-C^{14}	93	98	72	1.36
" "	39	45	36	1.25

* Data taken from Bloch and Rittenberg (1).

methyl and carboxyl carbons in the nucleus and side chain, the calculated ratio $(A):(B)$ for acetic acid-2-C^{14} is 0.84 and for acetic acid-1-C^{14} 1.26. Isotope analyses of greater precision are needed to establish more completely the accuracy of these ratios.

Isotope Concentration of Individual Carbon Atoms in Cholesterol—By isotope analysis of the angular methyl groups and of the isopropyl moiety of the isooctyl side chain, the origin of some individual carbon atoms of the cholesterol molecule has been determined. The choice of these carbon atoms was prompted by the relative ease of their isolation and by our interest in the biological synthesis of branched chain structures. Cholesterol synthesized from acetic acid-1-C^{14} and from acetic acid-2-C^{14} was used for this purpose.

TABLE III

Distribution of C^{14} in Isopropyl Group of Cholesterol Side Chain

Compound analyzed	Cholesterol formed from			
	$CH_3C^{14}OOH$		$C^{14}H_3COOH$	
	Found	Calculated	Found	Calculated
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Cholestane	93		71	
Acetone	62	70*†	72	85*§
Iodoform (C_{26}, C_{27})	20	0†	100	128§
Carbonyl carbon of acetone (C_{25})	146‡	209†	16‡	0§

* Calculated on the assumption that the methyl and carboxyl carbon atoms of acetic acid are present in cholesterol in a ratio of 15:12.

† Calculated from the C^{14} content of cholestane for 1 isotopic carbon (C_{25}) per molecule of acetone.

‡ Calculated from the observed values for acetone and iodoform.

§ Calculated from the C^{14} content of cholestane for 2 isotopic carbon atoms (C_{26}, C_{27}) per molecule of acetone. The predicted value of iodoform is 108 if the C^{14} concentration of C_{26} and C_{27} is calculated from the observed value for acetone (72 counts) from which the iodoform was obtained.

The formation of acetone and allocholanic acid on chromic acid oxidation of cholestane (5) constitutes the classical proof for the presence of a terminal isopropyl group in the aliphatic side chain of cholesterol. Hence, we have assumed that the acetone obtained by oxidation of cholestane is derived exclusively from carbon atoms 25, 26, and 27. The isotope concentrations were determined in acetone and in the iodoform derived from it, which represents C_{26} and C_{27}. The isotope content of the carbonyl atom of acetone (C_{25}) was calculated by difference. It is clear from the data in Table III that acetic acid had been utilized in the synthesis of the isopropyl group of cholesterol. When methyl-labeled acetic acid was the precursor of the steroid, the C^{14} content of the iodoform fraction (C_{26} and C_{27}) accounted for nearly all the C^{14} in the acetone. On the other hand, in cholesterol synthesized from carboxyl-labeled acetic acid, the methyl group of acetone had a very low radioactivity, showing that in this case the carbonyl group of acetone (C_{25}) contained most of the isotope. Carbon atoms 26 and 27 of cholesterol therefore originate from the methyl groups of acetic acid, while carbon atom 25 is derived from the carboxyl carbon of acetate (Fig. 1). It should be pointed out that in both experiments the C^{14} content of the "labeled" carbon atoms was about 15 to 20 per cent lower than was expected from the isotope concentration of the steroid and that those positions which one would expect to be unlabeled contained a small but significant radioactivity. These discrepancies may be attributed either to shortcomings of the degradative and analytical procedures or perhaps to a biological redistribution of labeled carbon during the biosynthesis. It is worthy of note that the formation of the isopropyl group in the side chain of cholesterol from the elements of acetic acid is the only known instance of the synthesis of a branched chain structure in the animal organism. All other constituents of the animal body which have branched carbon chains are either essential amino acids or vitamins.

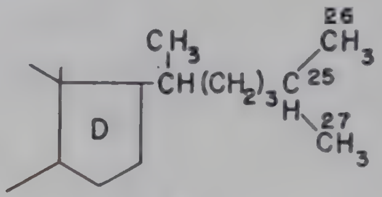

FIG. 1

Origin of Carbon Atoms 10, 17, 18, and 19—In the oxidation of organic compounds by a mixture of chromic and sulfuric acids, methyl groups and their adjoining carbon atoms are converted to acetic acid. This procedure has been made the basis for an analytical determination of carbon-bound methyl groups (6). We have applied this procedure to the nuclear hydrocarbon $C_{19}H_{30}$ which is obtained by thermolysis of cholesteryl chloride. If it is assumed that this hydrocarbon has the structural formula proposed by Bergmann and Bergmann (7), chromic acid oxidation should yield a maximum of 2 moles of acetic acid (degradation)[2] from the methyl carbon atoms 18 and 19 and their adjoining carbon atoms (C_{10} and C_{17}) (Fig. 2). The observed yield of acetic acid (degradation) was 1.3 to 1.5 moles. The acid was found to contain isotopic carbon both when methyl-labeled and carboxyl-labeled acetic acid had been the cholesterol precursor. The acetic acid obtained on chromic acid oxidation of the cholesterol nucleus is derived from 4 carbon atoms of the steroid. Therefore, the isotope concentrations observed for the methyl groups of acetic acid (degradation) reflect the average C^{14} content of carbon atoms 18 and 19, and those given for the carboxyl group of acetic acid[3] (degradation) represent the average concentration of carbon atoms 10 and 17 (Table IV). In cholesterol for which $CH_3C^{14}OOH$ had been the precursor, the angular methyl groups contained no C^{14}. On the other hand, the isotope concentration was high at carbon atoms 18 and 19 in cholesterol which had been synthesized from $C^{14}H_3COOH$, demonstrating that methyl groups of acetic acid are the source of the angular methyl groups of the steroid molecule. In Experiment I (Table IV), the isotope concentration of the acetic acid obtained

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[2] The acetic acid resulting from the degradation is designated as acetic acid (degradation) in order to distinguish it from the acetic acid which serves as a precursor in steroid synthesis and which is designated acetic acid (precursor).

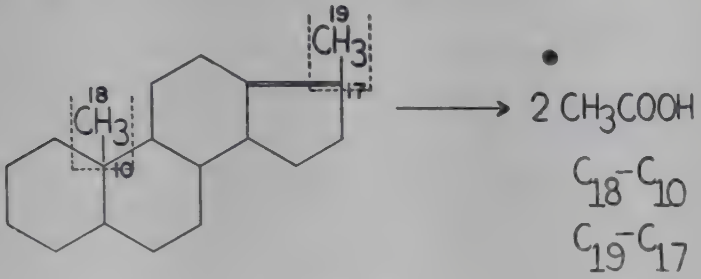

FIG. 2

TABLE IV

Isotope Concentration in Angular Methyl Groups and C_{10} and C_{17} of Cholesterol

Compound analyzed	Cholesterol synthesized from					
	$CH_3C^{14}OOH$ (Experiment I)			$C^{14}H_3COOH$ (Experiment II)		
	Found	Calculation A*	Calculation B*	Found	Calculation A*	Calculation B*
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*
1. Cholesterol	93			71		
2. Acetic acid by oxidation of $C_{19}H_{30}$	64	105†	53†	76	64†	96†
3. $CO_2(C_{10} + C_{17})$ from (2)	120	128	128	40	0	50
4. Iodoform from methyl carbons ($C_{18} + C_{19}$) of (2)	2	0	0	105	152	101

* The two methods of calculation (A and B) are explained in the text.

† Calculated on the assumption that the methyl and carboxyl carbon atoms of acetic acid are present in cholesterol in a ratio of 15:12. The calculated values in lines (3) and (4) are based on the observed C^{14} content of the acetic acid (line(2)).

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[3] It is assumed that the acetic acid (degradation) is formed in equal amounts from carbon atoms 18 and 10, and 19 and 17 respectively.

by degradation was entirely accounted for by the C^{14} content of its carboxyl group, and therefore either carbon atom 10 or 17, or both, must have been derived from carboxyl groups of the precursor acetic acid. It will be noted that in Experiment II (Table IV) the CO_2 fraction also had a significant radioactivity, which suggests that this fraction (C_{10} and C_{17}) had originated in part from methyl groups of acetic acid. It may further be seen that in Experiment I (Table IV) the acetic acid (degradation) had an appreciably lower isotope content and in Experiment II a higher isotope concentration than would have been expected if methyl groups of acetic acid (precursor) had been the source of carbon atoms 18 and 19 only, and if carboxyl groups of acetic acid had supplied both carbon atoms 10 and 17 (Calculation A). It should be recalled here that the methyl carbon 19 changes its position during the thermolysis of cholesteryl chloride and becomes attached to C_{17} (7). While one might expect both angular methyl groups in cholesterol to be attached to carbons which were originally carboxyl carbons of acetic acid, this need not be the case for C_{19} after it has migrated to C_{17}. Since, as indicated in Experiment II (Table IV), the mixture of C_{10} and C_{17} contained a significant isotope concentration, 1 of these 2 carbon atoms may have been derived from methyl groups of acetic acid, and it seems likely that C_{17} was the labeled one in this case. With these considerations in mind, we have calculated (Calculation B) the expected isotope concentrations of carbon atoms 10, 17, 18, and 19 and have assumed that, in Experiment I, of the 2 molecules of acetic acid formed on degradation, 1 contains no isotope and the other C^{14} in the carboxyl group (C_{10}), while in Experiment II 1 molecule of acetic acid is doubly labeled (C_{17} and C_{19}) and the other singly (C_{18}). The experimental data for the most part are in satisfactory agreement with this assumption.

Admittedly the procedures which have been employed in order to determine the isotope concentration of the angular methyl groups are not unequivocal. We have therefore carried out an independent degradation experiment to substantiate the above results. When cholesterol is heated in the presence of palladium-charcoal, about 4 moles of hydrogen and 1 mole of methane are evolved per mole of cholesterol (8, 9). According to Ruzicka *et al.* (9), the aromatization of Rings A and B of cholesterol causes the elimination of the angular methyl group (C_{18}) in the form of methane. When this reaction was applied to cholesterol which had been synthesized from $CH_3C^{14}OOH$, the methane produced had a low isotope concentration, but when cholesterol synthesized from $C^{14}H_3COOH$ was subjected to dehydrogenation, the methane contained 73 to 81 per cent of the expected radioactivity (Table V). These results taken together with those observed on chromic acid oxidation of the cholesterol nucleus ($C_{19}H_{30}$) strongly indicate that the angular carbon atoms 18 and 19 have their origin in methyl groups of acetic acid. The source of carbon atoms 10 and 17 is less certain, although it appears from the analytical data that one of them is derived from a carboxyl and the other from a methyl group of acetic acid. We conclude tentatively that C_{10} is furnished by a carboxyl carbon of acetic acid and C_{17} by a methyl group.

Previous considerations of the quantitative significance of acetic acid as a sterol precursor were based on experiments with intact rats (1, 2). These animals received deuterioacetate or C^{13}-acetate for a relatively short time, and it was computed that under these conditions acetate furnished about half of the carbon and hydrogen atoms of cholesterol. Since the calculation of these values included an estimate of the half life time of cholesterol and of the dilution of dietary isotopic acetate by acetic acid from endogenous sources, quantities which cannot be determined very accurately, the values given were considered to be approximate only. The results reported here on the isotope concentration of individual carbon atoms of the steroid molecule furnish independent evidence that 2-carbon compounds are the principal source of carbon in the biosynthesis of cholesterol. If acetic acid or an equivalent 2-carbon compound was the sole precursor, then any single carbon atom in the sterol which is synthesized from acetic acid labeled in one position only should contain either about twice[4] the isotope concentration of the total molecule or no excess isotope at all. On the other hand, if, for example, acetic acid supplied only half of all the carbon atoms, then any individual carbon of cholesterol should contain either

TABLE V

Radioactivity of Methane (C_{18}) Formed by Dehydrogenation of Cholesterol

	Cholesterol synthesized from	
	$CH_3C^{14}OOH$ (Experiment I)	$C^{14}H_3COOH$ (Experiment II)
	c.p.m.	*c.p.m.*
Cholesterol	92	59
Methane Sample 1*	13 (207)†	86 (106)†
" " 2	23	77
" " 3	32	80
" " 4	43	83

* See "Experimental" for explanation of Samples 1 to 4.

† The values in parentheses are the calculated isotope concentrations of a single carbon if derived from the labeled carbon of the precursor, corrected for the ratio of 15:12 for the ratio of methyl carbon to carboxyl carbon in cholesterol.

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[4] Since the methyl and carboxyl carbon atoms of acetic acid are not used equally in the synthetic process but in a ratio of 1.27, the isotope concentration of an individual carbon atom should be either 1.8 or 2.5 times that of the total steroid molecule.

about 4 times the isotope concentration of the total molecule or no excess isotope at all. Since the radioactivities at those carbon atoms which were analyzed individually were found to contain very little radioactivity when acetic acid labeled in one position was the precursor and contained close to the theoretical radioactivity in the case of the acetic acid labeled at the other carbon atom, it is probable that this will also be the case for the remainder of carbon atoms in the sterol molecule. The close agreement between postulated and observed isotope distribution is perhaps the most convincing evidence that a 2-carbon compound is the principal if not the sole building block of cholesterol.

EXPERIMENTAL

Preparation of Labeled Acetic Acid—Acetic acid-1-C^{14} was prepared from $C^{14}O_2$ and methyl magnesium bromide.

Acetic acid-1-C^{13} was obtained from acetonitrile, as described by Weinhouse *et al.* (10).

Acetic Acid-2-C^{14}—20 mM of methyl alcohol containing C^{14} were mixed with 15 ml. of hydriodic acid (sp. gr. 1.7) and a trace of red phosphorus, and heated under a reflux for 1 hour. A slow stream of nitrogen was passed through the reaction flask and the methyl iodide was collected in a trap cooled by dry ice. The methyl iodide (1.05 ml.) was transferred to a solution containing 1.01 gm. of sodium cyanide in 1.8 ml. of water and the mixture heated under a reflux for 6 hours.[5] Acetonitrile was distilled from the reaction mixture with several additions of water to the reaction flask. The distillate containing the acetonitrile was hydrolyzed by heating under a reflux with 10 per cent sodium hydroxide for 24 hours. For purification the solution was heated for an additional half hour in the presence of potassium permanganate. Acetic acid was obtained by steam distillation of the acidified solution. The yield based on methyl alcohol was 66 per cent.

Acetic Acid-2-C^{13}—C^{13}-methyl iodide[6] was allowed to react with KCN and converted to acetic acid as described for the preparation of acetic acid-2-C^{14}.

Preparation of Labeled Cholesterol—The isotopic cholesterol used in the present experiments was obtained by incubation of rat liver slices in the presence of isotopic acetate as described before (3). Tissue from young male rats of the Sprague-Dawley strain (60 to 100 gm. in weight) was employed, since it had been noted (3) that the incorporation of isotopic carbon into cholesterol was considerably faster in younger than in older animals. In a typical experiment, 1.5 gm. of liver slices were incubated in 12 ml. of Krebs' phosphate buffer, pH 7.4, which contained 3 mg. of appropriately labeled acetic acid. The flasks were shaken at 37° for 6 hours. The gas phase was pure oxygen which was replaced every 2 hours. Cholesterol was isolated by way of cholesterol digitonide in the usual manner. Under our experimental conditions, the isotope concentration or specific activity in the isolated cholesterol varied from 2 to 4 per cent of that of the acetate used. This permitted a several hundred fold dilution of the C^{14}-containing sterol by non-isotopic cholesterol, thus providing adequate amounts of marked sterol for degradation studies. In the experiments in which the utilization of the 2 carbon atoms of acetic acid for cholesterol synthesis was determined, appropriate mixtures of the differently labeled acetates were added to the incubation medium. Their isotope concentrations are given in Tables I to VI.

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[5] This procedure was worked out by I. Zabin in this laboratory.

[6] Furnished as a contribution by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

Degradation of Cholesterol into Nucleus and Side Chain—Cholesteryl chloride prepared from cholesterol was pyrolyzed as described before (1). It was assumed that the low boiling fraction was a mixture of isooctane and isooctene and represented the isooctyl side chain of cholesterol. This fraction was analyzed for isotope content after redistillation. The material remaining after removal of the volatile hydrocarbon was distilled *in vacuo* and yielded as the main fraction a hydrocarbon distilling at 185–230° at 1 mm. of Hg. The elementary composition and optical rotation of this material corresponded to those for the hydrocarbon $C_{19}H_{30}$ described by Bergmann and Bergmann (7).

$C_{19}H_{30}$. Calculated, C 88.4, H 11.6; found, C 88.3, H 11.7

The hydrocarbon obtained from two different runs had $[\alpha]_D^{25} = +30.3°$ and +33.5° (2 per cent in benzene) respectively. This hydrocarbon represents the intact steroid skeleton and is referred to in this paper as the cholesterol nucleus.

Oxidation of Hydrocarbon $C_{19}H_{30}$ by Chromic Acid—A mixture of 1 mM of hydrocarbon $C_{19}H_{30}$, 7.5 gm. of chromium trioxide, and 6.0 ml. of sulfuric acid was heated under a reflux for 3 hours. Under these conditions methyl groups and their adjacent carbon atoms are converted to acetic acid (6). The reaction mixture was then subjected to steam distillation, the acid in the distillate determined by titration, and acetic acid isolated as the silver salt. The yield of acetic acid from the oxidation of different samples varied from 1.30 to 1.47 moles per mole of hydrocarbon $C_{19}H_{30}$. The isotope concentration of the carboxyl carbon of this acetic acid (degradation) was determined either by decarboxylation of silver acetate with bromine in carbon tetrachloride (11) or by pyrolysis of lithium acetate at 350–400° to yield acetone and lithium carbonate (12). Analysis of iodoform obtained from this acetone yielded the isotope concentration of the methyl group of acetic acid (degradation). In order to test the reliability of the procedures for the degradation of acetic acid, control experiments were carried out with a sample of acetic acid-2-C^{14} of known specific activity. The results given in Table VI show that the degradation products have the expected isotope concentrations, except for iodoform which had a specific activity about 10 per cent lower than that calculated. No corrections have been made for this dilution in the values reported in Tables III and IV.

Dehydrogenation of Cholesterol by Palladium-Charcoal—0.40 gm. of isotopic cholesterol was intimately mixed with 0.15 gm. of 10 per cent palladium-charcoal and heated, after initial evacuation of the system, to 330–350° for 24 hours (8, 9). A gas trap cooled by liquid nitrogen was connected to the vacuum line in order to condense any hydrocarbons formed. After gas evolution had ceased, nitrogen was admitted to the system until atmospheric pressure had been reached. Nitrogen gas was passed slowly through the gas trap which was kept at −180°, and the exit gases were swept directly into a micro combustion furnace. Oxygen was fed into the combustion tube from a side arm. The combustion gases were passed through a $Ba(OH)_2$ solution in order to precipitate the carbon dioxide as $BaCO_3$. The baryta traps were changed every 20 minutes and the C^{14} content of the $BaCO_3$ samples measured separately (Samples 1 to 4, Table V). Since methane is the only hydrocarbon which has a sufficiently high vapor pressure at the temperature of liquid nitrogen, it is assumed that the gas removed is mainly methane. However, the varying isotope concentration of the different samples indicates that at least in Experiment I, Table V, the gas contained a second component.

TABLE VI

Degradation of Acetic Acid-2-C^{14}

	Found	Calculated
	c.p.m.	*c.p.m.*
$C^{14}H_3COOH$	2040	
CH_3COCH_3	2680	2720
CHI_3	3740	4080
CO_2*	20	0
CO_2†	0	0

* By thermolysis of lithium acetate.

† By decarboxylation of silver acetate with bromine.

Degradation of Cholesterol to Allocholanic Acid and Acetone—Cholesterol was converted to cholestane by way of dihydrocholesterol and cholestanone (13). The hydrazone of cholestanone was reduced to cholestane by the Wolff-Kishner method as described by Dutcher and Wintersteiner (14). The over-all yield from cholesterol was 52 to 57 per cent.

Oxidation of cholestane by chromic acid according to Windaus and Neukirchen (5) yields allocholanic acid and acetone. For the degradation of isotopic cholestane, 3 gm. of the steroid were suspended in 125 ml. of glacial acetic acid and 6.5 gm. of CrO_3 dissolved in 25 ml. of 80 per cent acetic acid were added in the course of 30 minutes. The reaction mixture was heated on the steam bath for 6 hours. Acetone was removed from the oxidation mixture by a slow stream of nitrogen and precipitated as the mercury complex (15) by passage through a boiling solution of mercuric sulfate. After completion of the oxidation, water was added to the flask and the reaction mixture was distilled to obtain the remaining acetone. The yield of acetone, calculated from the weight of the mercury-acetone complex, ranged from 10 to 20 per cent. Allocholanic acid was isolated according to the directions of Windaus and Neukirchen (5). The yield of crude potassium salt was about 10 per cent of the cholestane used. Purification of the allocholanic acid by recrystallization so diminished the yield as to prevent further use of this material for degradation studies. Attempts to increase the recovery by use of the procedure described by Fieser (16) for the oxidation of isoalkylnaphthoquinones were unsuccessful.

The C^{14} content of the acetone was determined by combustion of the mercury-acetone complex. For isotope analysis of the methyl groups of acetone, the mercury-acetone complex was decomposed by hydrochloric acid and the acetone converted to iodoform after distillation. The isotope content of the carbonyl group of acetone was calculated by difference.

Isotope Analyses—For C^{14} analysis all the samples were burned in a micro combustion apparatus, except the samples of mercury-acetone and iodoform, which were converted to carbon dioxide by the wet combustion procedure of Van Slyke and Folch (17). CO_2 was precipitated as barium carbonate and counted in a flow gas counter. Samples were counted for a sufficient length of time to insure less than 5 per cent probable error. The C^{14} values are given as counts of C^{14} per minute of $BaCO_3$ samples, corrected for infinite thickness. C^{13} was determined by mass spectrometric analysis.

SUMMARY

1. The biological synthesis of cholesterol has been investigated with the aid of acetic acid-1-C^{14}, acetic acid-2-C^{14}, and with acetic acid labeled by both C^{13} and C^{14}.

2. The utilization of the 2 carbon atoms of acetic acid as a carbon source for the total molecule and for the cholesterol nucleus and side chain has been determined.

3. Methyl groups of acetic acid have been identified as sources of carbon

atoms 18, 19, 26, 27, and presumably 17 in cholesterol. Carbon atom 25 and proabably carbon atom 10 are furnished by carboxyl groups of acetic acid.

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THE COLORIMETRIC ESTIMATION OF URINARY NEUTRAL STEROID ALCOHOLS*

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The neutral non-ketonic alcohol fraction of urinary lipide extracts constitutes a substantial portion of the total neutral fraction as now separated by conventional procedures. A knowledge of the variations in the excretion of the compounds in this fraction would supplement the information obtained by other analytical procedures such as the Zimmermann reaction. Few methods have been proposed for the quantitative estimation of these alcohols. Electrometric titration of half succinates or half phthalates has been utilized by Lieberman *et al.* (1, 2) and the less specific antimony trichloride reaction has been used by Pincus (3). It was believed that a colorimetric method specific for carbinols would be useful both for isolation studies and for clinical purposes.

The wide and successful use of the Zimmermann reaction for the estimation of steroid ketones suggested that it might serve as a model. The reagent chosen was 3,5-dinitrophthalic anhydride. This compound contains the potentially chromogenic *m*-dinitrophenyl group and is capable of reacting with primary and secondary alcohols to yield alkali-soluble half esters. These half esters developed a red color upon treatment with methanolic potassium hydroxide in the absence of a ketone. The chromogenic properties of *m*-dinitrophenyl compounds in alkaline solution have been known for many years (4, 5) but in most instances the colors developed have been too transient to be suitable for quantitative measurements. It was found possible to define conditions for the application of this reaction to the quantitative estimation of urinary neutral steroid alcohols.

Method

Urinary Extracts—Duplicate aliquots of the neutral fraction of a 24 hour specimen in absolute ethanol, each usually amounting to one-tenth of the

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total, are transferred to 10 ml. round bottom flasks and evaporated to dryness on the water bath with the aid of gentle suction. 1 ml. of toluene is added to each residue and removed by heating under gentle suction. After two more such treatments with toluene the residues are dried *in vacuo* over calcium chloride overnight. The size of the sample is adjusted if unusually high or low values are expected. Acetone solutions of pure steroid alcohols are simply evaporated to dryness in 10 ml. round bottom flasks immediately prior to esterification.

To each flask 24 mg. (100 μeq.) of dinitrophthalic anhydride and 0.2 ml. of pyridine are added. The flasks are stoppered loosely and heated on the boiling water bath for 15 seconds. After cooling, the reaction mixture is diluted with 10 ml. of 0.1 N hydrochloric acid and transferred to a 60 ml. separatory funnel with 10 ml. of ether. The mixture is extracted with four 5 ml. portions of ether. The combined ether extracts are then washed with three 5 ml. portions of 0.1 N hydrochloric acid and five 5 ml. portions of water. The ether solution is then transferred to a 50 ml. volumetric flask with 10 ml. of ether and made up to volume with absolute methanol.

3 ml. aliquots of the methanol-ether solutions of the ester are transferred to colorimeter tubes and the solvents evaporated by immersing the tubes in a boiling water bath. The last traces of solvents are removed by the application of gentle suction. The residue is then dissolved in 7.0 ml. of methanol and treated with 3.0 ml. of 5.00 N potassium hydroxide[1] in methanol. A standard solution containing 0.5 μeq. of cholesteryl hemi-3,5-dinitrophthalate is run with each set of unknowns. The readings are made at 510 mμ with a Coleman junior spectrophotometer 5 minutes after the addition of potassium hydroxide. The size of the aliquot taken for analysis should be adjusted so that a reading of 50 ± 10 per cent transmission is obtained.

Color Reaction

Cholesteryl hemidinitrophthalate was prepared by the action of dinitrophthalic anhydride upon cholesterol in the presence of pyridine, and thi ester was utilized for the study of the color reaction. Treatment of th ester with methanolic potassium hydroxide results in the immediate development of a pink color in the absence of a ketone.

The characteristics of the color reaction were then studied in detail in Coleman junior spectrophotometer. The spectral absorption and extinc tion coefficients[2] from 400 to 600 mμ of cholesteryl hemidinitrophthalate and dinitrophthalic acid are shown in Fig. 1. It may be seen from the curves that steroid hemidinitrophthalates, of which group the cholesteryl ester is typical, show maximal absorption at 510 mμ, while 3,5-dinitrophthalic acid has its maximum at 530 mμ and requires an alkali concentration higher than that of the esters to develop comparable extinction.

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[1] The methanolic potassium hydroxide is prepared in 4 liter batches and standardized against 1.0 N HCl. The titer of this solution remains constant for 2 to months when the bottle is protected by a soda-lime tube.

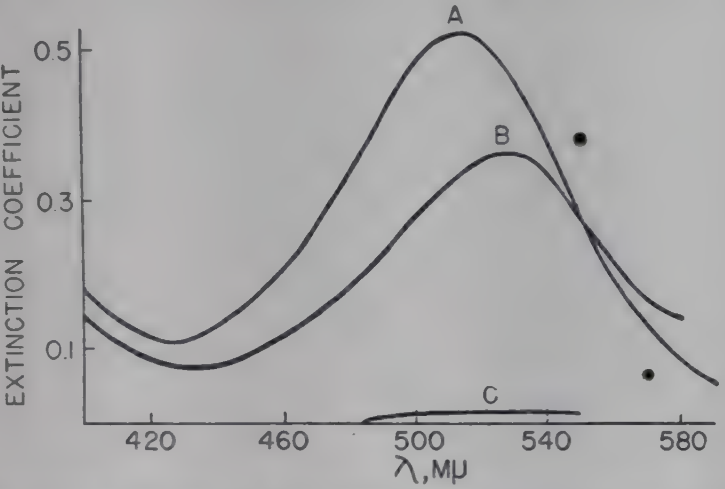

Fig. 1. Absorption spectra of cholesteryl hemi-3,5-dinitrophthalate in 1.52 N KOH in methanol (Curve A), 3,5-dinitrophthalic acid in 3.03 N KOH in methanol (Curve B), and 3,5-dinitrophthalic acid in 1.48 N KOH in methanol (Curve C).

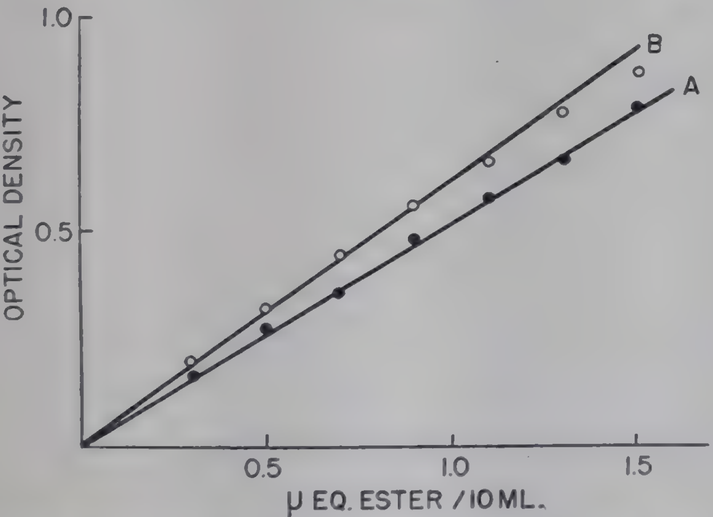

Fig. 2. Optical density-concentration relationships. Curve A, cholesteryl hemi-3,5-dinitrophthalate in 1.5 N KOH in methanol; Curve B, 2-methyl 3,5-dinitrophthalate in 1.5 N KOH in methanol.

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[2] The extinction coefficient as used in this report is defined as the optical density of a solution containing 1 μeq. of ester in 10 ml. of solution contained in a 7 × $\frac{7}{8}$ in. test-tube.

Beer's law is obeyed for all the compounds studied thus far, in the range 0.2 to 1.5 μeq. of ester, although in the case of 2-methyl 3,5-dinitrophthalate some deviation occurs above 1.0 μeq. (Fig. 2). The effect of alteration of the potassium hydroxide concentration on the extinction coefficient of

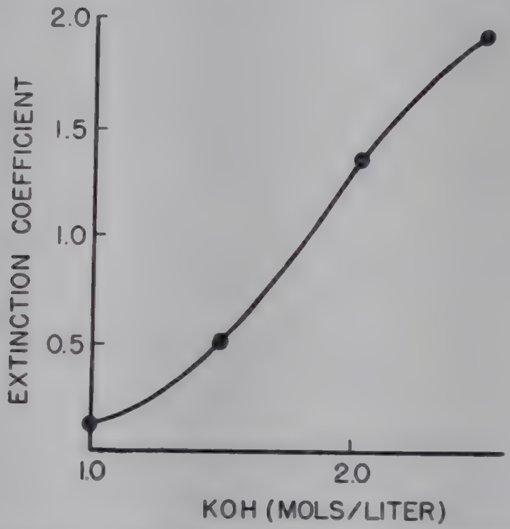

Fig. 3. Relation between extinction coefficient of cholesteryl hemi-3,5-dinitrophthalate and potassium hydroxide concentration.

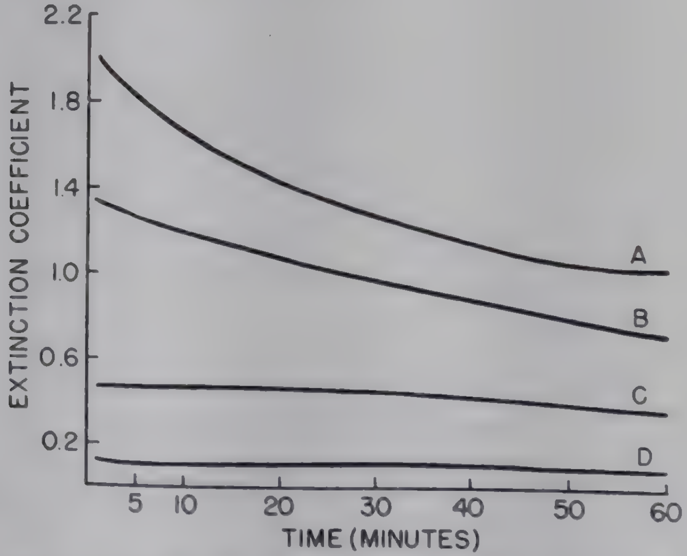

Fig. 4. The effect of potassium hydroxide concentration on the stability of the color developed by cholesteryl hemi-3,5-dinitrophthalate. Curve A, 2.49 N KOH; Curve B, 1.98 N KOH; Curve C, 1.47 N KOH; and Curve D, 1.02 N KOH.

cholesteryl hemidinitrophthalate is shown in Fig. 3. Although higher extinction coefficients may be obtained by increasing the potassium hydroxide concentration in the reaction mixture, the rate of fading of the color also increases, as can be seen from Fig. 4. The region of 1.5 N potassium hydroxide appears to represent a satisfactory compromise between sensitivity and color stability. At this concentration, the color has been found to be sufficiently stable so that readings may be made within 5 minutes after mixing the reagents.

TABLE I

Esterification of Cholesterol with 3,5-Dinitrophthalic Anhydride

10 μeq. of cholesterol were used in each experiment.

Variation	Anhydride	Pyridine	Heating time	Standing time	Esterification
	μeq.	*ml.*	*sec.*	*hrs.*	*per cent*
Reactants	100	0.2	15	48	94
	100	0.5	15	48	84
	100	1.0	15	48	84
	100	1.5	15	48	69
Reactants	20	0.2	15	0	71
	40	0.2	15	0	84
	50	0.2	15	0	88
	70	0.2	15	0	81
	80	0.2	15	0	97
	150	0.2	15	0	85
Heating time	100	0.2	5	0	84
	100	0.2	30	0	88
			min.		
	100	0.2	1	0	90
	100	0.2	5	0	86
			sec.		
Standing time	100	0.2	15	0	90
	100	0.2	15	1	87
	100	0.2	15	2	85
	100	0.2	15	4	84
	100	0.2	15	16	89
	100	0.2	15	24	85

Esterification of Pure Steroid Alcohols

In order to adapt the color reaction to analytical purposes, it was necessary to define conditions under which maximal esterification would proceed, the variables being time, temperature, and the proportions of the reactants. Experiments were performed to determine the extent of esterification of cholesterol at varying time intervals. When a 10-fold excess of 3,5-dinitrophthalic anhydride (100 μM) in 0.2 ml. of pyridine was used to esterify cholesterol, the reaction proceeded to its maximal extent almost instantly at room temperature, as indicated in Table I. Increasing the volume of pyridine reduced the yield of ester. By using 0.2 ml. of pyridine and the 15 second heating period to insure complete solution of the reactants, it was found that a 5-fold excess of anhydride over alcohol led to maximal yields of ester. Nevertheless a 10-fold excess of anhydride is usually used in esterifying both pure compounds and urinary extracts.

The processing of the reaction mixture and the preparation of the ester for colorimetric analysis were found to be important, since substantial errors may be introduced by failure to effect complete removal of dinitrophthalic acid. It was found that, if the reaction mixture is diluted with water containing sufficient hydrochloric acid to half neutralize the pyridine, the esters may be extracted quantitatively with ether. The ether extracts may then be freed from pyridine by washing with dilute acid, and residual dinitrophthalic acid removed by washing with water. Control experiments in which the steroid alcohol was omitted gave extracts which yielded no color with 1.5 N methanolic potassium hydroxide even when 400 μM of dinitrophthalic anhydride were used. Crystalline cholesteryl hemi-3,5-dinitrophthalate treated in a similar manner was recovered quantitatively.

TABLE II

Esterification of Steroid Alcohols with 3,5-Dinitrophthalic Anhydride

Compound	Esterification* and standard deviation
	per cent
Cholesterol	93 ± 3
Epicholestanol	69 ± 4
Androsterone	51 ± 5
Dehydroepiandrosterone	89 ± 3
Epiandrosterone	72 ± 5
Δ^5-Androstenediol-3β,17β	64 ± 5†
Androstanediol-3β,17β	68 ± 5†
Pregnanediol-3α,20α	77 ± 4†
17-Methyltestosterone	3

* Cholesterol hemidinitrophthalate was used as the standard.

† Based upon esterification of both hydroxyl groups.

The extent of esterification of a series of representative steroid carbinols was then studied, the conditions found optimal for the esterification of cholesterol being utilized. The yield of ester was based upon the extinction coefficient of pure cholesteryl hemi-3,5-dinitrophthalate which has been adopted as the reference standard. It will be seen from the data presented in Table II that in no case did the yield of ester reach the high values given by cholesterol. These results may be due to the failure of more hindered hydroxyl groups to react with the anhydride or possibly to destruction of the carbinols as a result of oxidative action of the dinitroanhydride. It is somewhat surprising that such variations in conditions as heating the reaction mixture for 1 hour, using rigorously dried pyridine, increasing the ratio of anhydride to alcohol, and substituting purified lutidine and quinoline for pyridine did not increase the extent of esterification of epicholestanol,[3] the behavior of which is typical of the unreactive carbinols. The one tertiary alcohol tested, 17-methyltestosterone, failed to react significantly with dinitrophthalic anhydride.

It is interesting that all the pure esters of 3,5-dinitrophthalic acid (Table III) which have been prepared by the action of the anhydride upon the alcohol have their maximal absorption at the same wave-length (510 mμ). The size of the alkyl substituent which has been varied from C_1 to C_{27} appears to have little effect upon the extinction at this wave-length. It is also noteworthy that the presence of a carbonyl group does not affect the wave-length of maximal absorption or the value of the extinction coefficient.

TABLE III

Crystalline Hemidinitrophthalates

The absorption maximum is 510 mμ.

Alcohol	Extinction coefficient	KOH
		moles per l.
Cholesterol	0.52	1.52
Epicholestanol	0.53	1.51
Dehydroepiandrosterone	0.58	1.52
Androsterone	0.49	1.50
Methyl*	0.62	1.51

* The structure of this ester is discussed in the text.

Esterification of Urinary Extracts

In order to determine the applicability of this procedure to the estimation of alcohols in urinary extracts, aliquots of a pooled neutral fraction were esterified with dinitrophthalic anhydride and the color developed in the usual manner. The spectral characteristics of the ester mixture were similar to those of the pure steroid esters, although greater absorption in the region 400 to 440 mμ was observed. It was therefore necessary to make a correction for the intrinsic color of the extracts. Adherence to Beer's law was satisfactory both for ester mixtures of pooled neutral fractions and for neutral fractions from individual subjects chosen at random. The esters obtained from urinary neutral fractions differ from those of the pure compounds in that the rate of fading of the color of the former was somewhat accelerated.

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[3] The nomenclature and stereochemical configuration of the steroid compounds are based upon the recommendations of Fieser and Fieser (6).

The range of steroid alcohol concentration over which the standard quantities of reagents would give reproducible results was examined and the results plotted in Fig. 5. It will be seen that maximal esterification was obtained when the ratio of anhydride to maximal alcohol determined was greater than 2.5. This contrasts with the behavior of cholesterol which required a 5-fold excess of anhydride for maximal esterification.

Since the neutral urinary extracts prepared in this laboratory are frequently stored in ethanol solution, it became necessary to devise a method for the complete removal of this solvent from the extract prior to esterification. A urinary extract was prepared without the use of ethanol and stored in toluene. It was found that when aliquots of this extract were evaporated to dryness three times with toluene under diminished pressure and stored overnight in a vacuum desiccator over calcium chloride, identical values for the steroid alcohol content were obtained with the extract alone and the extract after the addition of ethanol.

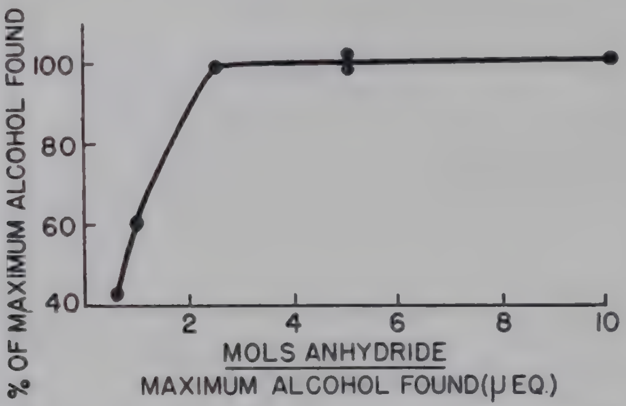

FIG. 5. The effect of varying the ratio of 3,5-dinitrophthalic anhydride to maximum alcohol determined in a urinary neutral fraction.

Application of Method

In order to determine whether this method would show changes in total urinary steroid alcohol excretion, 200 mg. of progesterone were administered to a male subject who had no obvious endocrinopathies. The daily excretion of ketosteroids (7) and steroid alcohols was followed for 7 days prior to the administration of progesterone. Progesterone was given intramuscularly in oil solution on 2 successive days. During these 2 days and the 2 following days, a marked increase in the excretion of steroid alcohols occurred (Fig. 6). In the last 3 days of the experiment the alcohol excretion dropped significantly below the pretreatment control level. Based

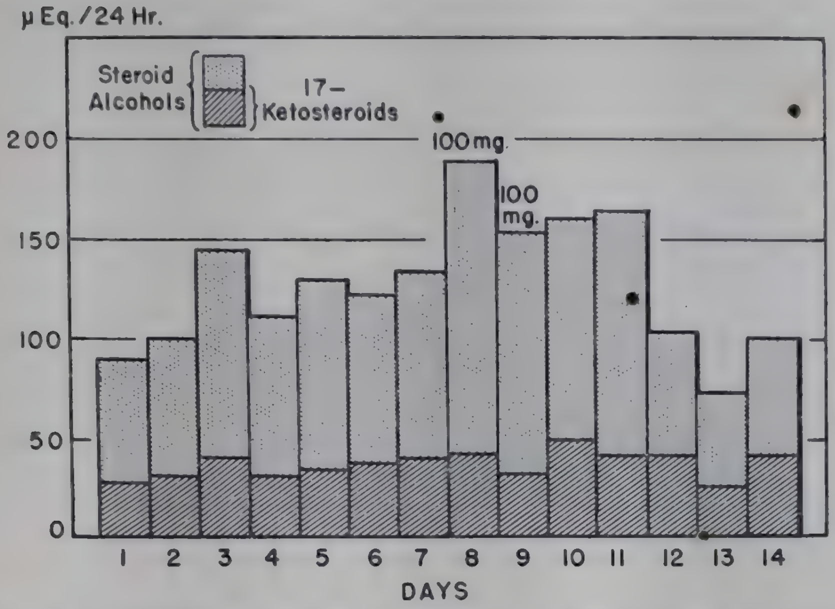

FIG. 6. The excretion of steroid alcohols and ketosteroids (microequivalents per day) by a normal male subject following the administration of two 100 mg. doses of progesterone.

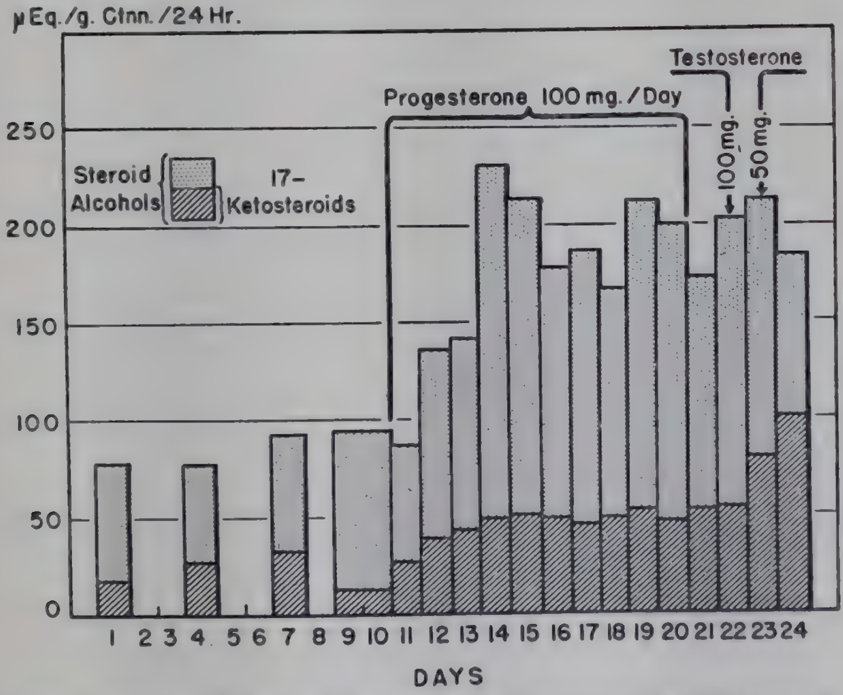

FIG. 7. The excretion of steroid alcohols and ketosteroids following the administration of progesterone and testosterone. The subject was a female with cancer of the breast. The excretion is expressed as microequivalents of ketosteroid and steroid alcohol per gm. of urinary creatinine per day.

upon the average pretreatment control level, the increased excretion corresponded to a recovery of 15 per cent of the administered progesterone calculated as pregnanediol.

A similar increase in the total urinary alcohol excretion was observed in a postmenopausal cancer patient treated with 100 mg. of progesterone daily for 9 days (Fig. 7) and testosterone for 2 days. In this instance there was a marked increase in the excretion of ketosteroids during the period of progesterone treatment. The significance of this change is not clear and is being studied. During the period of testosterone administration, the expected rise in ketosteroid excretion was observed, but on the final day of the experiment the non-ketonic alcohols appeared to drop to the control level, although the observation was discontinued too soon to be sure of this. The non-ketonic alcohol level was computed as the difference between the total alcohol value (microequivalents of cholesterol) and the ketosteroid value (microequivalents of dehydroepiandrosterone), assuming that equal numbers of carbonyl and alcohol groups are present in the ketosteroids.

TABLE IV

Urinary Excretion of Steroid Alcohols

Subjects*	Cholesterol
	μeq. per 24 hrs.
Men, normal	118
Women, normal (follicular phase)	115
" pregnant	→ 454
Addison's disease, man (1 subject)†	50
" " women (2 subjects)†	23
Panhypopituitarism, man	39
" women	22

* Unless otherwise indicated, the subjects were either laboratory personnel or patients under study by the staff of the Huntington Laboratory.

† We are indebted to Dr. George W. Thorn and Dr. Peter Forsham for the specimens from these patients.

This estimate is subject to error from two major sources, (1) the presence of non-alcoholic ketones in urine (artifacts of dehydration, etc.) which would tend to lower the apparent non-ketonic alcohol level and (2) the presence of non-steroidal alcohols and possibly amines which would give erroneously high values for the total alcohols. It is possible that a more accurate estimate of non-ketonic alcohol excretion would be obtained if the esterification with dinitrophthalic anhydride were carried out upon the non-ketonic fraction separated with Girard's reagent.

In Table IV are presented preliminary data on the excretion of steroid alcohols under various circumstances which indicate that the major source of the steroid alcohols is the adrenal cortex, with some contribution from the gonads. Further work on this problem is in progress and is directed toward an investigation of the excretion of steroid alcohols in normal subjects, patients with adrenal and gonadal malfunction, and cancer patients, particularly those treated with hormonal agents.

Structure of Esters

Theoretical considerations suggest that the steroid dinitrophthalates and the methyl dinitrophthalate prepared by a similar method are esters of the 2-carboxyl group. This view is supported by the properties of the isomeric 1-methyl ester prepared by the action of methanolic hydrogen chloride on dinitrophthalic acid. The behavior of these compounds and the dimethyl ester is summarized in Table V. In 1.5 N potassium hydroxide in methanol the 1-methyl ester color fades rapidly, but if the alkali concentration is doubled, the ester is converted quantitatively to dinitrophthalic acid within 15 minutes as measured by absorption at 530 mμ. In the cases of the 2-methyl and cholesteryl esters, in which the ortho effect is more pronounced, the fading is slower. If the ester solutions in 1.5 N potassium hydroxide are allowed to stand overnight, the colors are completely bleached. However, if the concentration of potassium hydroxide is then increased to 3 N, color corresponding to 65 per cent of the theoretical amount of dinitrophthalic acid (absorption maximum 530 mμ) is developed. The dimethyl ester which was prepared by the action of diazomethane upon dinitrophthalic acid gives an immediate deep orange-red color in 1.5 N potassium hydroxide in methanol. After a few moments the orange changes to the typical pink of the 2-methyl ester and the solution shows maximal absorption at 510 mμ.

TABLE V

Methyl 3,5-Dinitrophthalates

Ester	Preparation	Absorption maximum or color	
		1.5 N KOH in methanol	3.0 N KOH in methanol
1-Methyl......	Acid and methanol	Orange → colorless	Orange → 530 mμ
2-Methyl......	Anhydride and methanol	510 mμ	510 mμ
Dimethyl.....	Acid and diazomethane	Orange → 510 mμ	

EXPERIMENTAL

All melting points were measured on a Fisher-Johns instrument and were corrected unless otherwise stated.

Elementary analyses marked F. were semimicroanalyses performed by Dr. Carol K. Fitz, Melrose, Massachusetts. Those marked E. were microanalyses by the Elek Microanalytical Laboratories, Los Angeles, California.

The Coleman junior spectrophotometer was used for all spectrophotometric measurements.

Dinitrotetralin—Commercial grade tetralin was nitrated with fuming nitric acid and concentrated sulfuric acid by a modification of the method of Schroeter (8). The eutectic mixture of 1,2- and 1,3-dinitrotetralin, m.p. 71–74°, was obtained in 39 to 44 per cent yield. Material having this melting point was sufficiently pure for further treatment. This material has also been prepared by the nitration of tetralin with fuming nitric acid alone (9).

3,5-Dinitrophthalic Acid—Oxidation of the eutectic mixture of the dinitrotetralins by repeated treatments on the steam bath with 30 per cent nitric acid was carried out essentially as described by Schroeter (8). After recovery from the barium salt, the crude acid was crystallized from ether-ligroin (b.p. 60–90°) and then melted at 229–230° with decomposition in a sealed tube. By reoxidation of the acid mother liquors a yield of 50 per cent was obtained. Acid melting above 225° was sufficiently pure for conversion to the anhydride. The acid has also been prepared by the oxidation of 2-methyl-3,5-dinitrobenzoic acid with nitric acid in a sealed tube (10) and by the nitric acid oxidation of 2,4,5,7-tetranitronaphthol (11).

3,5-Dinitrophthalic Anhydride—The acid was dehydrated by refluxing with freshly distilled acetic anhydride as described by Eder and Widmer (10) and was obtained in about 75 per cent yield. The pure anhydride melted at 162–164° and had a neutral equivalent of 119. Only the first fraction was collected and the mother liquors were hydrolyzed with water and subjected to reoxidation.

1-Methyl 3,5-Dinitrophthalate—A solution of 4 gm. of 3,5-dinitrophthalic acid in 400 ml. of 1 per cent hydrogen chloride in methanol was boiled under a reflux for 12 hours. The solvent was removed under reduced pressure and the residue dissolved in a small volume of methanol and distilled to dryness. The oily residue was finally dried *in vacuo* over potassium hydroxide.

The separation of the ester from unchanged acid by means of countercurrent distribution in isopropyl ether-water was unsuccessful. The recovered mixture was taken up in ether and chromatographed on a column of magnesium silicate No. 16 (1 part)[4] and Celite No. 545 (3 parts) measuring 34 × 125 mm. Elution was carried out with benzene, benzene-ether mixtures, and ether. The material eluted by benzene was recrystallized five times from benzene-ether and once from chloroform. The pure 1-methyl ester obtained melted at 177–179°.

$C_9H_6N_2O_8$. Calculated, N 10.4, neutral equivalent 270
Found, " 10.3 (F.), neutral equivalent 267

2-Methyl 3,5-Dinitrophthalate—3,5-Dinitrophthalic anhydride was dissolved in absolute methanol and, after standing at room temperature for

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[4] Philadelphia Quartz Company, Berkeley, California.

1½ hours, the solution was evaporated under reduced pressure. The residue was crystallized twice from ether-pentane mixture and yielded pure ester which melted at 178–179° and depressed the melting point of 1-methyl dinitrophthalate.

$C_9H_6N_2O_8$. Calculated, N 10.4; found, N 10.4 (F.)

Dimethyl 3,5-Dinitrophthalate—The reaction of 3,5-dinitrophthalic acid with excess diazomethane yielded the dimethyl ester which melted at 126–126.5° after four crystallizations from ether.

$C_{10}H_8N_2O_8$. Calculated, N 9.9; found, N 9.8 (F.)

Cholesteryl Hemi-3,5-dinitrophthalate—A mixture of 1.93 gm. (5 mM) of cholesterol and 11.90 gm. (50 mM) of dinitrophthalic anhydride was dissolved in 25 ml. of pyridine by heating for about 30 seconds on the water bath. After cooling, the reaction mixture was diluted with 475 ml. of water and 25 ml. of concentrated hydrochloric acid and extracted with 300 ml. and four 100 ml. portions of ether. The ether extracts were washed with nine 100 ml. portions of 0.1 N hydrochloric acid and five 100 ml. portions of water. After adding 25 ml. of benzene, the ether solution was evaporated to dryness under reduced pressure. The residue was recrystallized five times from aqueous acetone to yield 2.49 gm. of cholesteryl hemidinitrophthalate, m.p. 215–216°, with softening and darkening at 206°.

$C_{35}H_{48}N_2O_8$. Calculated, N 4.5; found, N 4.4, 4.6 (F.)

Epicholestanyl Hemi-3,5-dinitrophthalate—A mixture of 390 mg. (1 mM) of epicholestanol and 2.38 gm. (10 mM) of 3,5-dinitrophthalic anhydride was dissolved in 10 ml. of pyridine by heating on the boiling water bath for 15 seconds. After cooling, the flask was stoppered and allowed to stand at room temperature for 24 hours. The reaction mixture was then diluted with 145 ml. of water and 5 ml. of concentrated hydrochloric acid and extracted with three 50 ml. portions and one 30 ml. portion of ether. The ether solution was washed with three 30 ml. portions of 0.1 N hydrochloric acid and five 30 ml. portions of water, then diluted with 25 ml. of benzene. After evaporation to dryness, the residue was recrystallized four times from aqueous acetone and three times from aqueous methanol. Epicholestanyl hemi-3,5-dinitrophthalate melts at 211–212.5° with softening at 205°.

$C_{35}H_{50}N_2O_8$. Calculated, N 4.5; found, N 4.4 (F.)

Dehydroepiandrosterone Hemi-3,5-dinitrophthalate—A mixture of 144 mg. of dehydroepiandrosterone and 1.9 gm. of 3,5-dinitrophthalic anhydride was dissolved in 7 ml. of pyridine and allowed to stand at room temperature for 22 hours. The reaction mixture was then diluted with 70 ml. of 2.5 N hydrochloric acid and extracted with 40 ml. and then with four 25 ml. portions of ether. The combined ether extracts were washed with four 25 ml. portions of 0.1 N hydrochloric acid, then with three 25 ml. portions of water, dried over sodium sulfate, and evaporated to dryness.

The product was further purified by an eight-transfer counter-current distribution in separatory funnels with 20 per cent ethyl acetate in hexane as the upper phase and 50 per cent ethanol as the lower phase. The volume of each phase was 40 ml. At the end of the distribution 15 ml. of absolute ethanol were added to each funnel to make the system homogeneous. After measurement of the volume, aliquots were removed from each funnel and analyzed for ester. The contents of Funnels 2, 3, and 4 which contained the bulk of the ester were combined and evaporated to dryness under reduced pressure. The crystalline residue was recrystallized twice from aqueous ethanol and the product melted at 222–224° with decomposition.

$C_{27}H_{30}N_2O_9$. Calculated, N 5.32; found, N 5.43 (E.)

Preparation of Androsterone Hemi-3,5-dinitrophthalate and Recovery of Androsterone from Reaction Mixture—A mixture of 100 mg. (0.34 mM) of androsterone and 785 mg. (3.4 mM) of dinitrophthalic anhydride was dissolved in 7 ml. of pyridine and heated on the water bath for 15 seconds. After cooling, the reaction mixture was diluted with 100 ml. of water containing 3.5 ml. of concentrated hydrochloric acid and extracted with 60 ml. and four 35 ml. portions of ether. The combined ether extracts were washed three times with 0.1 N hydrochloric acid and five times with water, dried over sodium sulfate, and taken down to dryness. Analysis showed that 43 per cent of the androsterone had been esterified. The residue was extracted with six 10 ml. portions of boiling hexane and filtered. The hexane solution was evaporated to a small volume and the crystalline precipitate which formed was filtered off and dried. 4.5 mg. of material, m.p. 184°, were obtained. No depression of the melting point was observed upon mixture of this material with an authentic specimen of androsterone.

The hexane-insoluble material was recrystallized from methanol and yielded 36 mg. of androsterone hemidinitrophthalate, m.p. 227–228°, with decomposition.

$C_{27}H_{32}N_2O_9$. Calculated, N 5.30; found, N 5.14 (E.)

SUMMARY

1. The action of methanolic potassium hydroxide upon certain steroid hemidinitrophthalates gives colored products which are of sufficient stability to be useful for quantitative measurements.

2. Secondary alcohols of the steroid group may be converted to chromogenic esters in 51 to 93 per cent yield by the action of 3,5-dinitrophthalic anhydride in pyridine.

3. This reaction has been applied to the estimation of urinary steroid alcohols and has yielded information concerning the origin and amounts of these compounds present in urinary extracts.

4. The anticipated increase in urinary steroid alcohol excretion was observed following the administration of progesterone to a normal male subject and to a female cancer patient.

We are indebted to the Schering Corporation for the samples of androstanediol and androstenediol.

We wish also to express our thanks to Miss Gladys Ekman for the ketosteroid determinations reported.

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LACTIC DEHYDROGENASE OF LIVER AND ITS RELATION TO THYROID ACTIVITY IN THE RAT*

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(Received for publication, August 8, 1949)

The relationship between the thyroid hormone and enzyme activity has been studied in this work. Changes in metabolic rate have been reported by various investigators to parallel certain changes in enzyme activity.

Thus enzyme studies on liver homogenates from thyrotoxic rats have shown an increase in cytochrome oxidase, succinoxidase (1), and D-amino acid oxidase activities (2). A decrease in the concentration of these enzymes and of cytochrome *c* (3) was noted following thyroidectomy. Since lactic acid metabolism seems to be disturbed in hyperthyroidism, as evidenced by increased blood lactic acid values and decreased lactate tolerance (4–7), the present work was undertaken to find out whether changes in liver lactic dehydrogenase activity could be observed similar to those reported for the above oxidative enzymes. Lactic acid oxidation has been studied for the most part in heart muscle systems (8–12), but data have also been reported for liver and other organs (10, 13).

In an approach to this problem, it was found that optimum conditions had not yet been described for a study of liver lactic dehydrogenase. In this report such conditions are presented in conjunction with a comparison of data obtained from normal and from (1) thyroparathyroidectomized, (2) thiouracil-treated, and (3) thyroid-fed rats.

EXPERIMENTAL

Male rats[1] which had been fasted for 16 to 24 hours were stunned by a blow on the head, decapitated, and exsanguinated. The liver was removed rapidly, placed on ice, and appropriate portions weighed directly into a homogenizing tube. 4 volumes of ice-cold redistilled water were added and the mixture homogenized for 4 minutes in a Potter-Elvehjem homogenizer (14). The resulting homogenate was filtered through cheesecloth and centrifuged in a clinical centrifuge at 2500 R.P.M. for 4 minutes. The supernatant liquid was diluted with water to give an approximately 3 per cent extract and was used in all further work, except in early experiments when a comparison of whole *versus* centrifuged homogenates was made. All the enzyme activity was found to be in the supernatant liquid. The dry weights of all samples were determined by evaporation of the extract to constant weight.

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* From a thesis submitted by Augusta A. Knoepfelmacher in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois, August, 1948.

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[1] The albino rats used were obtained from the University of Illinois inbred colony and from Sprague-Dawley, Inc., Madison, Wisconsin.

Oxygen uptake was measured by means of the conventional Warburg apparatus at 38°. All the reactants except diphosphopyridine nucleotide (DPN) were added to the main compartment of the flask, which was kept at room temperature before being placed in the bath. DPN solutions were pipetted into the side arm of the Warburg flasks and added to the main compartment after a 5 minute equilibration period.

All acidic components of the system were adjusted to pH 8.0 with sodium hydroxide, except DPN which was adjusted to pH 7.0. The components of the final reaction mixture were as follows: 0.5 ml. of 3.5×10^{-4} M cytochrome *c*, 0.3 ml. of a 1 per cent solution of DPN (50 per cent purity), 0.4 ml. of 0.2 M sodium phosphate buffer or 0.8 ml. of 0.1 M sodium diethyl barbiturate (veronal) at pH 8.0, 0.8 ml. of 0.4 M DL-lactate, 0.2 to 0.4 ml. of a 3 per cent rat liver extract, and water to make a total volume of 3 ml. (0.2 ml. of 0.25 M nicotinamide was also added in a number of experiments.)

Cytochrome *c* was prepared from frozen horse heart according to the method of Keilin and Hartree (15) and standardized photometrically after momentary heating to 95° and filtering and discarding any precipitate formed. DPN was prepared from bakers' yeast according to the method of LePage (16). Nicotinamide was a Merck product, and the lactic acid used was either the Mallinckrodt (N. F. VIII, 85 per cent, sp. gr. 1.206) or Coleman and Bell (U. S. P., 85 per cent) product. The concentrated lactic acid was diluted with redistilled water, boiled to hydrolyze lactides and lactic anhydrides, and neutralized with sodium hydroxide. The sodium lactate solution must be made up from concentrated lactic acid at least every 4th day. On standing in the refrigerator, lactate solutions allow bacterial growth, which results in the conversion of lactic acid to pyruvic acid; the latter inhibits lactate oxidation (10). Meyerhof and Lohmann (17) have shown that in tissues L-lactic acid is oxidized at the same rate as DL-lactic acid; that is, the presence of the unnatural isomer does not inhibit the utilization of the natural isomer.

Pyruvate analyses were performed colorimetrically, according to the method outlined in Umbreit *et. al.* (18), on trichloroacetic acid filtrates of the incubation mixture. Lithium pyruvate was used in the preparation of the standard curve.

Thyroid preparations were obtained from The Maltine Company. One sample of thyroglobulin contained 0.82 per cent total iodine and 0.18 per cent thyroxine iodine; another preparation of desiccated thyroid material contained 0.87 per cent total iodine and 0.20 per cent thyroxine iodine.

The data are expressed as $Q_{O_2}^{10}$ values. $Q_{O_2}^{10}$ values are calculated on the basis of microliters of oxygen consumed per 10 minutes per mg. of dry weight of tissue, and this value is multiplied by 6 to give the potential oxygen consumption per hour. Oxygen uptake was based on the first 10 minute period of incubation, except in a few experiments in which the uptake was greater in the second 10 minute period. After 20 minutes the oxygen consumption falls off slowly owing to the inhibitory action of the end-product, pyruvic acid.

All rats were 2 months old at the beginning of experiments. Hypothyroidism was produced by either thyroparathyroidectomy or by feeding the rats stock diets (Purina fox chow) containing 1 per cent thiouracil. Hyperthyroidism was elicited by feeding stock diets containing various amounts of thyroid-active material. The treated animals were fed *ad libitum*. In the case of thyroidectomized rats, two control groups of the same age were used: one group of rats was fed *ad libitum;* the other group was pair-fed with the thyroidectomized rats. The animals were used 4 months after removal of the thyroid gland. For thiouracil and thyroid-fed rats, normal males served as controls; they had free access to food. (At first pair-fed controls were used with the thiouracil group. But since it was shown later that rats which had been on a limited food intake for several months had the same lactic dehydrogenase activity as normal rats, pair-feeding of this group was discontinued.)

Results

The reversible oxidation of lactate to pyruvate in liver homogenates is assumed to proceed by interaction of lactate with DPN on the surface of the lactic dehydrogenase. Subsequent steps then involve cytochrome reductase, cytochrome *c*, cytochrome oxidase, and oxygen.

The oxidation of malate to oxalacetate proceeds through similar steps (13). The reoxidation of reduced DPN through the cytochrome system has been discussed by Potter in connection with the malic dehydrogenase system (13); he has shown that DPN and cytochrome *c* have to be added in excess to liver homogenate systems, while cytochrome reductase and cytochrome oxidase are already present in excess. If this is the case in the system described in the present report, then lactic dehydrogenase becomes the rate-limiting enzyme. This could be proved conclusively by adding graded amounts of pure liver lactic dehydrogenase to a system and observing the changes in oxygen uptake; however, the enzyme has not yet been purified. But since it is reasonable to assume that the reoxidation of reduced DPN proceeds through the same steps whether lactic or malic acid is the hydrogen donor, the imposition of malate oxidation on lactate oxidation should show whether lactic dehydrogenase is the rate-determining enzyme. The addition of malate and glutamate to the lactic dehydrogenase system increased the oxygen uptake in several experiments by about 60 per cent. (Glutamate was added to remove the end-product of malate oxidation, oxalacetate, by transamination (13).)

Table I

Optimum Conditions for Liver Lactic Dehydrogenase System

The data for each compound added were obtained from separate experiments. 0.4 ml. of 3 per cent liver extract was added per flask; the other components of the reaction mixture were added as described in the text.

DL-Lactate		Cytochrome *c*		DPN		Nicotinamide		Phosphate	
Molarity	$Q_{O_2}^{10}$	Molarity $\times 10^{-5}$	$Q_{O_2}^{10}$	Molarity $\times 10^{-4}$	$Q_{O_2}^{10}$	Molarity	$Q_{O_2}^{10}$	Molarity	$Q_{O_2}^{10}$
0	2.0	0	2.0	0	4.0	0	83.9	0*	93.0
0.01	39.0	1.03	42.8	0.25	32.7	0.008	80.5	0.027	87.3
0.024	59.0	3.10	71.2	1.26	58.1	0.017	74.4		
0.049	68.5	4.12	73.3	2.52	64.3	0.034	80.9		
0.098	86.7	6.20	73.8	3.71	73.3				
0.194	81.9			8.0	73.8				
0.267	79.7			12.6	72.6				

* Veronal buffer was used (0.027 M).

The $Q_{O_2}^{10}$ values in the fourth and sixth columns were obtained in early experiments in which lactate solutions were used after more than a week's storage. This results in diminished oxygen uptake, as explained in the text.

The optimum concentrations of the components of the system have been determined, and the data are condensed in Table I. The need for added cytochrome *c* and DPN is clearly indicated. The quality of the DPN added is of considerable importance. DPN not purified by the LePage procedure (16) gave high and irregular blank values (oxygen uptake in the absence of lactate); in the presence of purified DPN consistently low blank values were obtained. Mann and Quastel (19) reported that DPN is rapidly destroyed in tissue homogenates and that nicotinamide inhibits this breakdown. Potter (13) made use of this fact by adding nicotinamide to the malic dehydrogenase system. Under the conditions used here, no decrease in oxygen uptake could be observed in the absence of added nicotinamide even after a 60 minute incubation period (Table I). As indicated in Table I, inorganic phosphate is not a necessary component of the system; veronal buffer is equally effective. Since Fig. 1 indicates that the optimum pH is about 8.0, veronal, which is a better buffer at this pH than phosphate, is to be preferred in the system. Fig. 1 illustrates the necessity of careful pH control. If the determinations had been carried out at pH 7.0, for example, the slightest variations in final pH values would have resulted in changes in oxygen uptake not related to the state of the animals. At about pH 8.0, small changes in pH values do not result in appreciable changes in oxygen consumption.

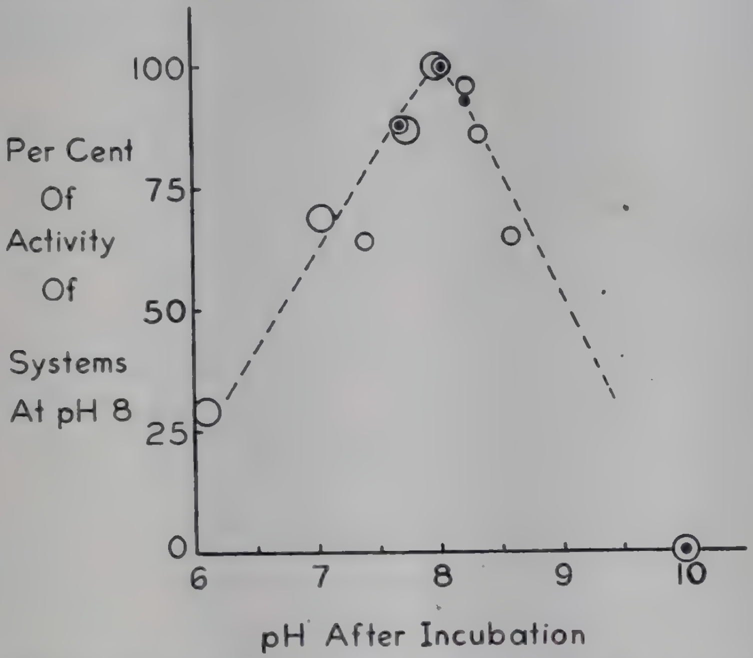

Fig. 1. Determination of the optimum pH for the liver lactic dehydrogenase system. The components of the reaction mixture are described in the text. Phosphate and veronal buffers were used. The data were obtained from four separate experiments. ○, normal rats, veronal buffer; ○, thiouracil-fed rat, phosphate buffer; ●, hyperthyroid rat, phosphate buffer.

Fig. 2 shows a direct proportionality between the oxygen uptake of the system and the tissue concentration when 1 to 4 mg. of tissue (dry weight) is added per flask. The data indicate the suitability of this system for tissue assay.

In order to correlate oxygen consumption with lactate utilization, pyruvate analyses were performed at the end of a 20 minute incubation period. Added pyruvate is not oxidized in the system. The results demonstrate that for 1 mole of oxygen consumed slightly more than 2 moles of pyruvate are formed (2.2 to 2.5 moles). The high pyruvate values obtained can probably be explained. During the time (1 to 2 minutes) aliquots of the homogenate were transferred from the Warburg flask into trichloroacetic acid, oxygen consumption continued, but was not recorded.

Since pyruvate accumulates in the system and is known to inhibit lactate oxidation (Table II), glutamate was added to effect the removal of pyruvate through transamination. This principle has been used successfully to remove oxalacetate in the assay for malic dehydrogenase (13). The addition of glutamate to the complete system increases the oxygen uptake (Table II) in an irregular fashion. The end-product of the proposed transamination reaction, α-ketoglutarate, is not utilized in the system. Das (20) reported that sodium fluoride inhibits heart lactic dehydrogenase activity. As Table II shows, the effect of sodium fluoride on liver lactic dehydrogenase activity was found to be negligible.

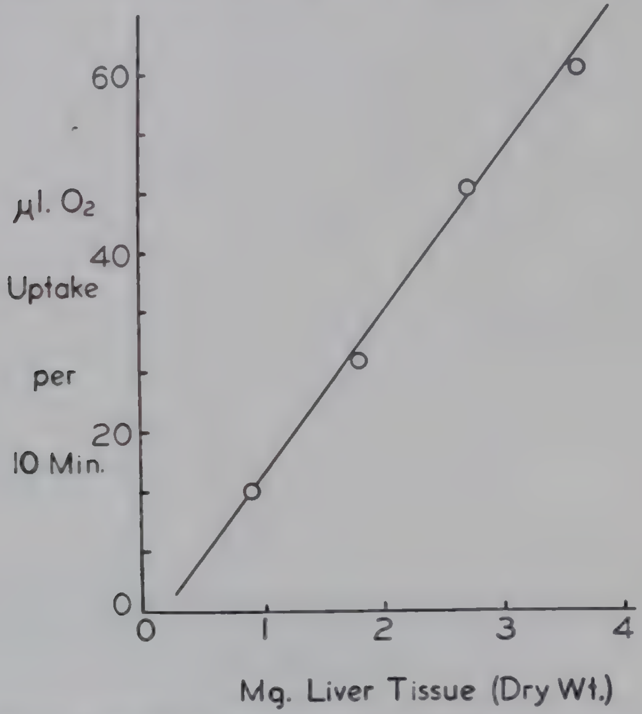

FIG. 2. Relationship between oxygen uptake and tissue concentration in the rat liver lactic dehydrogenase system. The components of the reaction mixture are described in the text, except that 0.1 to 0.4 ml. of 3 per cent liver extract was used.

Many conflicting experiments are reported in the literature on the addition of thyroglobulin and thiouracil *in vitro*. In Table II, it is shown that the addition of either thyroglobulin or thiouracil *in vitro* does not affect the oxygen uptake.

Fig. 3 shows the rate of lactate oxidation in the system. Oxygen uptake is linear with respect to time for the first 20 minute period and then decreases slowly. In the absence of the substrate, lactate, oxygen consumption is negligible. The addition of glutamate, α-ketoglutarate, or pyruvate in place of lactate does not increase the oxygen uptake of the system significantly above that of the blank. Pyruvate is not oxidized, even when magnesium ions and adenosine triphosphate are added to the components of the reaction mixture.

TABLE II

Effect of Adding Certain Substances on Oxygen Uptake of Liver Lactic Dehydrogenase System

The components of the reaction mixture are described in the text.

Experiment No.	Addition to complete system	Final concentration	$Q_{O_2}^{10}$
		M	
73	None		82.7
	Sodium fluoride	0.013	77.1
103	None		82.1
	Sodium fluoride	0.013	84.4
	" pyruvate	0.001	68.3
87*	None		57.8
	Sodium pyruvate	0.013	15.5
	" pyruvate	0.027	7.8
104	None		80.2
	Sodium L-glutamate	0.05	100.2
97†	None		98.2
	Sodium L-glutamate	0.067	137.4
79	None		79.2
	Thiouracil	0.001	83.4
82a	None		88.0
	Thyroglobulin	0.3%	86.4

* Thyroid-fed rat.

† Thiouracil-fed rat.

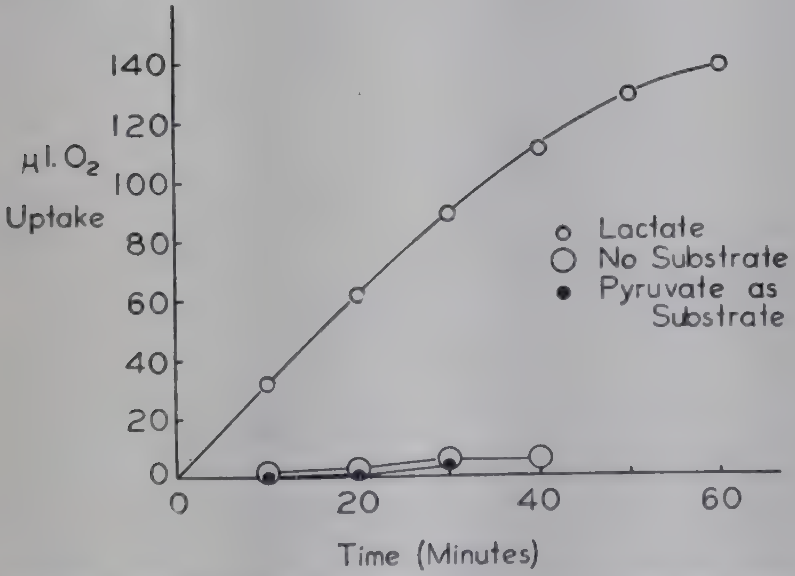

FIG. 3. Oxidation of lactate in the rat liver lactic dehydrogenase system. The components of the reaction mixture are described in the text. 1.8 mg. of liver (dry weight) were added per flask.

A satisfactory procedure for the assay of lactic dehydrogenase activity in liver homogenates enabled us to study the relation to thyroid activity.

Table III summarizes the results obtained with differently treated rats. There were no significant differences in enzyme activity among thyroidectomized, normal, and pair-fed control animals of the same age. Thiouracil treatment, however, resulted in a small, but statistically significant increase in enzyme activity.

Since it is doubtful whether the thyroid gland can be removed completely, the question may be raised whether the rats had been properly thyroidectomized. Although preliminary basal metabolic studies indicated that this was the case, changes in body and organ weights further demonstrated the striking decrease in growth following the removal of the thyroid gland. Thiouracil administration resulted in a similarly decreased growth rate, decreased food consumption, and decreased spleen weights; liver weights were the same as for normal rats. Thyroid treatment, on the other hand, was accompanied by an increased food consumption, a decrease in body weight in all but one case, and no noticeable change in organ size. There is no apparent correlation between the amount (0.2 to 1.0 per cent of the diet) and nature (thyroglobulin or desiccated thyroid) of the administered thyroid material and liver lactic dehydrogenase activity.

TABLE III

Comparison of Liver Lactic Dehydrogenase Activity in Normal and Treated Rats

Treatment	No. of animals*	$Q^{10}_{O_2}$		t†	P
		Mean ± S.D.	Range		
None (age, 2½ mos.)	11 (19)	84.3 ± 3.6	78.4–93.6		
Thiouracil-fed	4 (11)	96.0 ± 3.0	92.8–99.8	5.4	<0.01
Thyroid-fed	7 (12)	67.7 ± 5.9	57.8–73.2	6.4	<0.01
None (age, 6 to 7 mos.)	3 (9)	87.0 ± 7.1	84.7–96.6		
Thyroidectomy	4 (7)	87.6 ± 3.0	82.8–91.0		
Pair-fed	2 (3)	89.4 ± 1.8	87.6–91.2		

* The figures in parentheses indicate the total number of animals used, including preliminary experiments which, when analyzed statistically, yielded similar comparative results.

† *t* values were calculated according to Chambers (21) and *P* values were obtained from the tables compiled by Fisher (22).

Liver homogenates of thyroid-fed rats showed a 22 per cent decrease in lactic dehydrogenase activity (Table III), which was found to be statistically significant. There is a difference of 42 per cent between thyroid-fed and thiouracil-fed rats.

DISCUSSION

The increased basal metabolic rate of the body accompanying hyperthyroidism seems to be reflected in an increased activity in enzyme systems so far investigated. The results obtained here appear to offer a contrast. However, on the assumption that the assay of enzymes in tissue homogenates reflects what occurs in the intact organism, the changes reported here can be correlated with results obtained by entirely different experimental approaches to the thyroid problem.

A literature survey pertinent to the relationship between hyperthyroidism and lactic dehydrogenase activity reveals the following facts: (1) Experimental hyperthyroidism is characterized by a marked liver glycogen deficiency (23–29). (2) One source of liver glycogen is lactic acid (30–33), and the mechanism of glycogen formation is believed to consist of a reversal of the steps involved in the glycolytic process (34). (3) Lactate utilization is disturbed in hyperthyroidism, as evidenced by high resting and working blood lactic acid values and decreased lactate tolerance (4–7). (4) Lactate utilization is also disturbed in serious clinical liver damage; this is the basis of the lactate tolerance test for liver function (35–37). (5) Clinical hyperthyroidism is frequently accompanied by liver damage (38).

A correlation of the results obtained in this paper with the observations cited above supports the hypothesis that the low liver glycogen, high blood lactic acid, and decreased lactate tolerance accompanying hyperthyroidism are at least to some extent due to the decreased capacity of the liver to synthesize glycogen from lactic acid.

It cannot be argued, however, that general liver damage is responsible for the decrease in enzyme activity, since succinoxidase, cytochrome oxidase (1), and D-amino acid oxidase (2) have been found to be increased in liver tissue of thyrotoxic animals. Furthermore, an increase in oxygen consumption of intact liver slices from thyroid-fed rats has been repeatedly demonstrated. It is possible that under the influence of excess thyroid hormone there is a general increase in non-specific oxidation in the liver at the expense of a specialized liver function; namely, glycogenesis from lactic acid.

SUMMARY

1. An assay for rat liver lactic dehydrogenase has been reported and the properties of the system studied.

2. A study has been made of the activity of rat liver lactic dehydrogenase at different levels of thyroid function. Thyroid administration resulted in a statistically significant decrease in enzyme activity. Thiouracil administration was accompanied by a small increase, and thyroidectomy did not affect the rate of lactate oxidation in the liver homogenates.

3. Experimental evidence has been reviewed in support of the hypothesis that hepatic glycogenesis from lactic acid may be disturbed in hyperthyroidism.

The authors wish to thank W. J. Frajola for the preparation of cytochrome *c*; Dr. A. V. Nalbandov, Department of Animal Science, University of Illinois, for a supply of thiouracil; Dr. G. E. Phillips of The Maltine Company for samples of desiccated thyroid and thyroglobulin; and Dr. F. J. Rudert of the Red Star Yeast and Products Company for making quantities of fresh bakers' yeast available to us.

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THE DETERMINATION OF GLUTAMIC ACID DEHYDROGENASE IN TISSUE HOMOGENATES*

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In 1938, two groups of workers published extensive studies of glutamic dehydrogenase in animal tissues. The enzyme preparation of von Euler, Adler, Günther, and Das (1) was a dialyzed extract from acetone-dried pig or beef liver. The preparation of Dewan (2) was the precipitate obtained at pH 4.6 from an extract of acetone-dried pig liver or kidney. Both studies showed approximately the same results. The preparations with L-glutamate and diphosphopyridine nucleotide (DPN) reduced methylene blue or took up oxygen when a suitable carrier was present. The final reaction products were shown to be ammonia and α-ketoglutarate with α-iminoglutarate as an intermediate product. The reaction was found to be reversible and the enzyme in question was shown to be highly specific for L-glutamic acid.

The present paper deals with the adaptation of the known facts of this enzyme system to tissue homogenates.

EXPERIMENTAL

Material and Methods—The animal tissues used in this study were obtained from young male rats of the Holtzman strain, approximately 3 to 4 months of age. The rats were killed by cervical dislocation and the tissue sample quickly taken, blotted, weighed, and placed in a cold homogenizing tube containing 0.5 ml. of water. The tissue was homogenized and sufficient cold glass-distilled water was added to give a 5 per cent homogenate. Aliquots of the homogenate were added to the properly fortified substrate in Warburg flasks with no side arms, which were kept cold until the tissue was added. The cold flasks were then attached to the manometers and transferred to the 38° bath and the measurement of oxygen uptake was begun after a 6 minute equilibration period.

The glutamic acid and semicarbazide hydrochloride were obtained from the Eastman Kodak Company. The latter was further purified by dissolving in hot water and recrystallizing with the addition of 2 to 3 volumes

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of cold alcohol. Cytochrome *c* was prepared in this laboratory according to the method of Keilin and Hartree (3) and was standardized photometrically (4). The DPN was prepared according to the method of LePage (5) and was 72 per cent pure as determined by spectrophotometric analysis. We are indebted to Dr. G. A. LePage for a sample of 85 per cent pure triphosphopyridine nucleotide (TPN) which was used in this study. All reagents, except cytochrome, were employed as the neutral potassium salts.

TABLE I

Optimum Conditions for Glutamic Dehydrogenase System

The source of enzyme was rat liver; 0.2 ml. of a 5 per cent water homogenate was added to each flask. The data shown for each compound added were the average of two experiments with a freshly prepared homogenate. Each compound was studied at varying concentrations with all other compounds held constant and at the optimum value. The concentration of each component selected for the assay technique is indicated by bold-faced type. The reaction volume of each flask was 3.0 ml. The Q_{O_2} was calculated on the basis of the two best consecutive 10 minute periods.

Glutamate		Phosphate		H^+ ions		DPN		Cytochrome *c*		Semicarbazide	
Molarity	Q_{O_2}	Molarity	Q_{O_2}	pH	Q_{O_2}	Mg. per flask	Q_{O_2}	Molarity × 10^{-5}	Q_{O_2}	Molarity	Q_{O_2}
0	4.0	0	50.6	6.0	16.0	0	0	0	8.0	0	43.1
0.033	41.2	0.0033	51.3	6.6	33.3	2	20.4	0.65	28.9	0.025	40.9
0.067	47.3	0.01	49.0	7.0	42.2	4	38.6	1.32	49.1	0.05	43.6
0.100	**49.7**	**0.02**	**47.8**	**7.2**	**43.0**	**6**	**50.4**	**2.64**	**48.9**	**0.075**	**49.9**
0.133	51.2	0.027	48.3	7.4	41.6	8	50.0	4.00	49.2	0.100	48.2
0.150	48.2	0.04	43.4	8.0	31.7			5.32	48.1		

Water versus Isotonic Homogenates—In previous studies, water homogenates have been used successfully in the determination of the succinic dehydrogenase and cytochrome oxidase system (6), of the glycolytic system (7), and of the malic system (8). On the other hand, isotonic homogenates have been demonstrated to be superior to water homogenates in the oxidation of oxalacetate (9) and the oxidation of octanoate (10). The average *Q* values of two representative determinations of glutamic dehydrogenase were 48.3 for water homogenates and 36.1 for isotonic homogenates. On this basis, water homogenates were used throughout this study.

Components of System—The study of the optimum concentrations of the various components of this system is summarized in Table I. The optimum pH was found to be 7.3 as reported by Dewan (2), who used pyocyanine as the carrier. The system does not appear to need additional sources of inorganic phosphate, but an appreciable amount was used in order to increase the buffering capacity of the medium. The data demonstrate the need for each of the other components, and the effect of each can be explained on the basis of the accompanying diagram in which Reactions A, B, and C are catalyzed by enzymes which are furnished by the homogenate, and are considered to represent glutamic dehydrogenase, DPN-cytochrome *c* reductase, and cytochrome oxidase respectively.

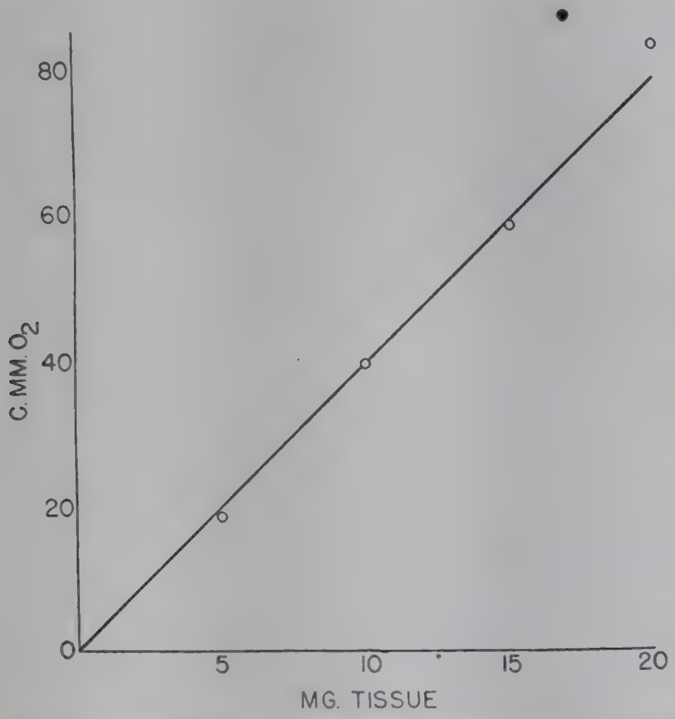
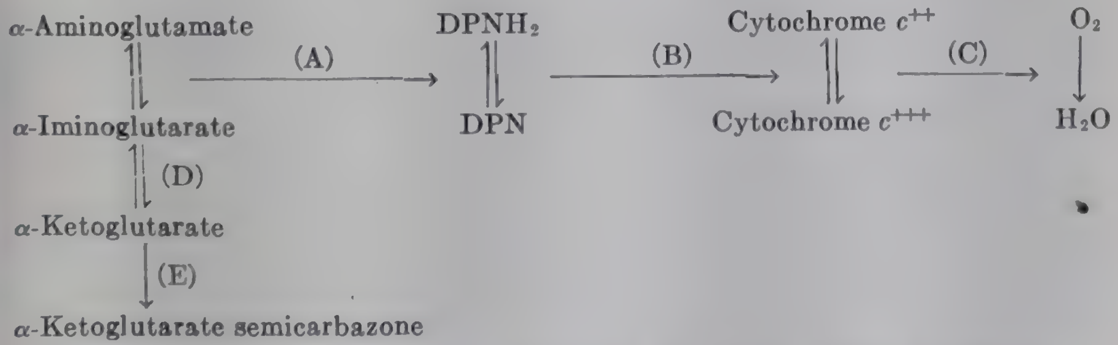


FIG. 1. Effects of different amounts of liver homogenate on oxygen consumption during a 20 minute period. The complete reaction mixture is as described in the text.


α-Aminoglutamate
(A)
DPNH₂
(B)
Cytochrome c⁺⁺
(C)
O₂
α-Iminoglutarate
DPN
Cytochrome c⁺⁺⁺
H₂O
(D)
α-Ketoglutarate
(E)
α-Ketoglutarate semicarbazone

Enzymes in the homogenate determine the rate of oxygen consumption when the critical components listed in Table I are present in excess. Proof that this is true is presented in Fig. 1, in which the rate is shown to be proportional to the amount of homogenate added. If one of the enzymes is definitely the limiting component, Fig. 1 shows that the system is probably valid for assay work. Previous assays for DPN-cytochrome *c* reductase (8) and cytochrome oxidase (6) show that these enzymes are present in rat liver in larger amounts than required here and are not limiting. Thus glutamic dehydrogenase appears to be the limiting enzyme in the system described in this paper.

The addition of nicotinamide at various concentrations to the reaction mixture had no beneficial effects on the enzyme system under study. The inclusion of semicarbazide as one of the components of this system is of questionable necessity, as shown in Table I. Dewan (2) has shown, however, that semicarbazide aids in maintaining the rate of reaction over a period of time. Our results confirm this observation. The theoretical need for the semicarbazide is apparent in the light of the results of von Euler *et al.* (1) which show that the equilibrium between α-amino- and α-iminoglutarate markedly favors the former. They also showed that significant concentrations of either ammonia or α-ketoglutarate, or small concentrations of both, inhibited the reduction of DPN by glutamic dehydrogenase. Therefore, the removal of one of the products of the oxidative deamination of glutamate by means of Reaction E will allow Reaction D, which is non-enzymatic, to proceed toward the formation of the keto acid. Thus, the removal of the keto acid tends to overcome the inhibitory effect of the unfavorable equilibrium on the dehydrogenation of glutamate. The semicarbazide also prevents the further metabolism of the α-ketoglutarate. This gives assurance that the oxygen consumption of the reconstituted system is a measure of the dehydrogenation of glutamate only and not the sum of this step plus the oxidative steps leading from α-ketoglutarate.

That the equilibrium between the amino and imino acids favors the formation rather than the breakdown of the amino acid may, in part, explain why such large quantities of DPN are necessary for optimum activity. It is apparent that the fixation of the end-product, the high substrate concentration, and the high concentration of DPN are all necessary to drive Reaction A in the desired direction at the maximum velocity.

DPN versus TPN—The two earlier studies (1, 2) both demonstrated that glutamic dehydrogenase is a coenzyme-linked enzyme. Both laboratories showed that DPN could serve as the coenzyme for the oxidation of glutamate. Neither group was able to demonstrate the oxidation of glutamate with TPN (2, 11) although it has been shown (1) that reduced TPN, as well as reduced DPN, readily facilitates the formation of glutamate from ammonia and α-ketoglutarate. A comparison of the effectiveness of the two pyridine nucleotides in the enzyme system studied here is shown in Table II. The data clearly indicate that TPN is less than 10 per cent as effective as DPN at comparable concentrations.

Recently, Mehler, Kornberg, Grisolia, and Ochoa (12) demonstrated that TPN and DPN worked equally well as the coenzyme for glutamic dehydrogenase. These workers used the spectrophotometric reduction of the pyridine nucleotide as the measure of activity. In this laboratory, with whole homogenates as the enzyme source, the spectrophotometric determination of the reduced coenzyme likewise showed that both pyridine nucleotides will react equally well with glutamic dehydrogenase. Thus, it is evident that the inability of TPN to serve as a coenzyme in the manometric determination of glutamic dehydrogenase is not due to a coenzyme specificity of the dehydrogenase but rather to an inability of reduced TPN to link with the cytochrome system. Horecker (13) has recently isolated a purified TPN-cytochrome *c* reductase from pig liver. Such an enzyme, if present in the rat liver used in these studies, was not present in sufficient

TABLE II

Comparison of TPN and DPN

Source of enzyme, 0.2 ml. of a 5 per cent rat liver homogenate. The amount of coenzyme indicated is calculated as *pure* coenzyme. All the conditions are optimum except for the coenzyme. The data are expressed as Q_{O_2}.

Pyridine nucleotide	DPN	TPN
mg. per flask		
1.5	22.8	0.0
3.0	46.0	1.5
4.5	59.3	5.1

TABLE III

Glutamic Dehydrogenase Assay of Various Rat Tissues

The tissues were prepared for study in the form of 5 per cent water homogenates and were added at a level of 10 mg. of tissue per flask, with all conditions optimum. The data are expressed in terms of Q_{O_2}.

Tissue	No. of samples	Glutamic dehydrogenase, average and range
Liver	43	49.3 (37.2–62.6)
Kidney	3	24.7 (19.3–29.1)
Heart	3	4.9 (0.0– 8.0)
Spleen	2	5.2 (3.5– 6.8)
Brain	3	10.3 (6.2–15.5)

concentration in the dilute homogenate to be effective in linking reduced TPN to the cytochrome *c* to any noticeable extent.

Assay Method and Results—On the basis of the foregoing studies the optimum reaction medium finally selected for the assay of glutamic dehydrogenase consists of the following: water to make a final volume of 3.0 ml., 0.3 ml. of 0.2 M potassium phosphate (pH 7.2), 0.6 ml. of 0.5 M potassium glutamate, 0.6 ml. of 1 per cent K-DPN (72 per cent pure), 0.2 ml. of 4×10^{-4} M cytochrome *c*, 0.2 ml. of 1.0 M semicarbazide (added just before the tissue), and 0.2 ml. of a 5 per cent water homogenate. The center well contained 0.1 ml. of 10 per cent KOH.

A number of assays have been carried out on rat liver and the results, together with data from other rat tissues, are presented in Table III. The relative activities of the various tissues compare closely with those obtained by von Euler *et al.* (1) from extracts of rat tissues. Arbitrarily assigning liver the activity of 100, these workers found the activity ratio of liver to kidney to brain to be 100:41:6. With the method outlined in the present study, the activity ratio for these three tissues was found to be 100:50:21. The demonstrable activity of brain homogenates again confirms the findings of Weil-Malherbe (14) and subsequent workers that brain tissue possesses the ability to metabolize glutamic acid.

SUMMARY

1. A method has been described for the assay of glutamic dehydrogenase in animal tissues.

2. The proposed technique is based on the measurement of the rate of oxygen uptake of water homogenates in a properly fortified medium by use of the conventional Warburg apparatus.

3. It has been demonstrated that DPN and not TPN is the necessary coenzyme for the manometric method proposed here. It is probable that the lack of oxygen uptake in the presence of TPN is due to a lack of TPN-cytochrome *c* reductase and not to a coenzyme specificity of the dehydrogenase, since spectrophotometric determinations have demonstrated the reduction of TPN by glutamic dehydrogenase.

4. Assays of various rat tissues are reported.

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THE DIABETOGENIC EFFECT OF DEHYDROASCORBIC AND DEHYDROISOASCORBIC ACIDS*

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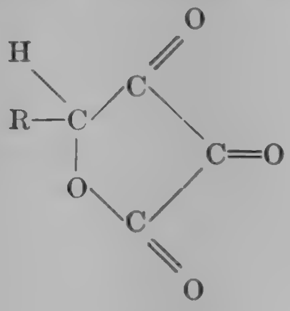
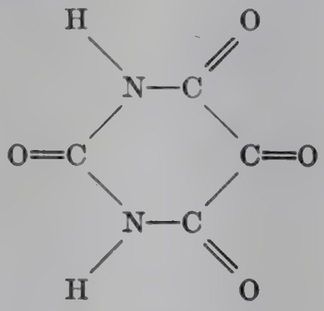
Ascorbic acid is known to exist in the animal body in a reversibly oxidized form as dehydroascorbic acid (I). Its chemical structure in the non-hydrated state is similar to that of alloxan (II).



(I)



(II)

The chemical properties of these two substances are strikingly similar. Both compounds are irreversibly decomposed under biological conditions. Alloxan has a half life of 1 minute (1) and dehydroascorbic acid has a half life of a few minutes (2). Alloxan is readily reduced to dialuric acid (3) with which it forms alloxantin (3). Dehydroascorbic acid is readily reduced to ascorbic acid (4) and also forms a similar intermediate compound (5). Alloxan (6) and dehydroascorbic acid (7) both form addition compounds with molecules containing the sulfhydryl group. Alloxan (8) and dehydroascorbic acid (9) both produce the Strecker reaction with amino acids and give the ordinary ketone reactions.

It would appear logical, therefore, to expect that there might be a relationship between alloxan and dehydroascorbic acid with regard to the production of diabetes. There is some evidence suggesting that dehydroascorbic acid may have diabetogenic properties. It is known that the dehydroascorbic acid level in the scorbutic animal is higher than normal, the dehydroascorbic acid-ascorbic acid ratio being as much as 20 times the normal value (10). Correlated with this is the fact that scorbutic guinea pigs have a decreased glucose tolerance (11) and show degeneration of the islets of Langerhans (12). Furthermore, ascorbic acid is known to act synergistically with alloxan (13, 14) and it seems probable that this effect is the result of the formation of dehydroascorbic acid. This is shown in the present work. It is also demonstrated that dehydroascorbic and dehydroisoascorbic acids produce diabetes in a manner similar to alloxan.

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* Aided in part by a grant from the Cleveland Diabetic Fund.

EXPERIMENTAL

Reagents—

Alloxan (Eastman).

Crystalline ascorbic Acid (Merck).

Dehydroascorbic acid was prepared immediately before each series of injections by dissolving ascorbic acid (1.08 gm.) in water (10 cc.) and shaking for 15 minutes with an equal volume of ether containing freshly sublimed quinone (0.66 gm.) (15). The ether layer was removed and the water solution was washed five times with an equal volume of ether. The excess ether was removed by suction and the solution used without further treatment. The yield is known to be excellent (16) and the solution was assumed to have about 100 mg. of dehydroascorbic acid per cc. The product was free of ascorbic acid by spectrophotometric examination and contained from 95 to 98 mg. of dehydroascorbic acid per cc. by polarimetric analysis. Slow decomposition produces a product with the opposite sign of rotation; thus these values are probably slightly low (17).

Dehydroisoascorbic acid was prepared from isoascorbic acid[1] by the procedure described above.

Action of Dehydroascorbic Acid on Rats—In male rats weighing 100 to 150 gm. the injection of 10 to 50 mg. of dehydroascorbic acid produces a characteristic reaction. Immediately after intravenous injection the rat becomes excited and runs aimlessly but rapidly around the cage for about 2 minutes. From time to time during this period, it may raise itself on its hind legs and vigorously rub its face with its fore legs. After this period of hyperactivity the rat collapses. It gradually starts to gasp for breath and slowly resumes normal respiration. There may be a slight serous discharge from the nose and mouth during the period of prostration. There is usually a brownish red discharge apparent around the eyes. With a dose of 20 mg. the animals appear normal about 10 minutes after the injection.

With larger doses the rats may not recover after the collapse. In a series of male Sprague-Dawley rats, 116 to 124 gm. in weight, that were injected intravenously with dehydroascorbic acid, there were no deaths in seven given 20 mg., one death in four given 30 mg., four deaths in eight given 40 mg., and four deaths in six given 50 mg. In the rats that died the heart continued to beat after respiration had ceased. At autopsy it was noted that there was no frothing in the trachea, that a section of lung would float in water, and that the right ventricle of the heart was dilated with blood, whereas the left ventricle was small.

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[1] Kindly donated by Dr. H. G. Luther of Chas. Pfizer and Company, Inc.

The characteristic hyperactive reaction could be produced with dehydroascorbic acid prepared by the oxidation of ascorbic acid with sodium hypochlorite, indicating that the effect was not due to an organic contaminant.

It is of great interest to note that the rats which had recovered from one injection of dehydroascorbic acid immediately tolerated a second dose several times the size of the initial dose, up to a maximum of about 1 gm. per kilo. This increased tolerance was noted as early as 10 minutes and as late as several hours after the administration of the initial dose. After a large second dose it was possible to give a similar large dose as much as a week later without killing the animal. The exact limits of this tolerance have not yet been determined. When the initial dose of dehydroascorbic acid was relatively large, there was little or no hyperactive response following the second dose. The rats became lethargic but occasionally they would bite an adjacent rat or object. With large doses respiration frequently became difficult. In those rats that died the autopsy findings were the same as those in rats that died following the initial dose. When the initial dose of dehydroascorbic acid was small, there was a second hyperactive response following the second dose. A small initial dose would not permit the administration of a large second dose.

Dehydroisoascorbic acid produced a similar hyperactive reaction in the rat, but a larger dose was required to produce the same effect and the lethal dose was much higher. As much as 160 mg. could be given as an initial dose with few deaths occurring. With this substance the animals did not tolerate a second dose that was greatly different from a large initial dose.

Synergistic Action of Dehydroascorbic and Ascorbic Acids with Alloxan—Male rats of Albino Farms stock were injected intravenously with alloxan without previous fasting and then, by the same needle, with ascorbic acid or dehydroascorbic acid. Diabetes was determined by the 48 hour blood sugar as in previous ascorbic acid studies (13, 14). Blood sugars over 150 mg. per cent were considered an indication of diabetes. When ascorbic acid was given with the alloxan, an alloxan dose of 20 mg. per kilo was necessary to produce diabetes in 44 per cent of the rats, whereas when dehydroascorbic acid was given with alloxan, an alloxan dose of 15 mg. per kilo was sufficient to produce diabetes in 58 per cent of the rats (Table I). Ascorbic acid with this smaller dose of alloxan had no effect on the level of blood sugar.

Diabetogenic Action of Dehydroascorbic and Dehydroisoascorbic Acids—

Blood sugars of male Sprague-Dawley rats were measured by a micromethod (18) before and from 2 to 14 days after intravenous injection of the test substances (Table II). The maximum dose of dehydroascorbic acid that could be given after an initial small dose, administered 10 to 30 minutes earlier, was about 1.1 gm. per kilo. In the rats that survived the injections hyperglycemia developed on the 3rd day and persisted for 3 days. 1 gm. per kilo of dehydroisoascorbic acid produced essentially the

TABLE I

Synergistic Action of Ascorbic Acid and Dehydroascorbic Acid with Alloxan after Intravenous Administration in Male Rats

No. of rats	Weight		Alloxan	Dehydroascorbic acid	Ascorbic acid	Diabetic		Non-diabetic	
	Range	Average				No. of rats	Average 48 hr. blood sugar	No. of rats	Average 48 hr. blood sugar
	gm.	*gm.*	*mg. per kg.*	*mg.*	*mg.*		*mg. per 100 cc.*		*mg. per 100 cc.*
6	102–154	117	30	20		6	392		
4	120–158	130	30		20	4	334		
4	96–144	112	20	20		4	375		
9	100–134	121	20		20	3	190	6	126
12	96–118	106	15	15		7	242	5	113
12	88–116	104	15		15			12	118
2	98–108	103	10	15				2	108
2	102	102	10		20			2	122
7	138–156	150	0	20				7	126

TABLE II

Diabetogenic Action of Dehydroascorbic and Dehydroisoascorbic Acids after Intravenous Administration in Male Rats

No. of rats	Weight		Dehydroascorbic acid	Dehydroisoascorbic acid	Average blood sugar before and after injection, mg. per 100 cc.								
	Range	Average			0 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	14 days
	gm.	*gm.*	*gm. per kg.*	*gm. per kg.*									
4*	116–120	118	1.1		110	125	330	182	156	132	130	133	
4	130–138	133		1.0	138	327	312	208	127	107	141	137	
2	130	130		1.5	137	400	450	450	450	450	450	450	450
4	120–126	123		1.4	116	246		357			371		327
2†	150–163	156		1.5	104	332		400			425		450

* 40 mg. were given each rat as a preliminary dose 10 to 30 minutes before the indicated dose.

† Fasted overnight.

same temporary hyperglycemia as did dehydroascorbic acid. The injection of 1.4 gm. per kilo or more of dehydroisoascorbic acid produced a permanent diabetes. One rat, observed for 4 months, maintained a blood sugar of over 400 mg. per cent and four rats showed a similar high blood sugar level after 6 weeks. A number of rats with hyperglycemia died within 2 weeks; one died on the 17th day and two after 6 weeks. Terminally, the blood sugar values tended to drop.

The effect of repeated doses of dehydroascorbic acid is shown in Table III. On the 1st day an initial dose of 20 mg. was administered intravenously 10 to 30 minutes before the first of three or more daily intravenous injections of 80 mg. Mortality during this period of injection was about 25 per cent. Of fourteen rats surviving a series of three or four injections, seven died with elevated blood sugars within 3 weeks, the highest mortality occurring about a week after the completion of the injections. The results on seven surviving rats along with one that received more injections are reported in Table III. At the end of 3 weeks there was a marked hyperglycemia in five of the eight rats. Smaller doses produced less striking results. However, one rat that received 60 mg. daily for 9 days showed an irregular hyperglycemia and a marked decrease in tolerance to glucose 40 days after the last injection. The intraperitoneal administration of 3.5 gm. of glucose per kilo produced an immediate rise in blood sugar which was maintained above 450 mg. per cent for 3 hours. The hyperglycemia resulting from daily injections of dehydroascorbic acid responded to the injection of small amounts of insulin.

TABLE III

Diabetogenic Action of Repeated Intravenous Injections of Dehydroascorbic Acid in Male Rats

Weight of rat	Dehydroascorbic acid*	No. of daily injections	Blood sugar before and after last injection, mg. per 100 cc.					
			Initial	2–3 days	4–5 days	6–7 days	14 days	21 days
gm.	*mg. per day*							
119	60–80	10	148	450	400	410	310	375
112	80	3	129	450	450	450	450	450
149	80	3	127	435	380	315	400	176
113	80	3	87	450	140	185	113	100
127	80	3	123				390	395
126	80	3	145				280	270
128	80	4	141	450	450	450	450	450
154	80	4	114	430	330	355	257	108

* On the 1st day an initial dose of 20 mg. was injected intravenously 10 to 30 minutes before the indicated dose.

DISCUSSION

A characteristic hyperactive reaction is produced in the rat by the intravenous injection of dehydroascorbic acid, and to a lesser degree by the injection of dehydroisoascorbic acid. Dehydroisoascorbic acid results from the oxidation of isoascorbic acid, which is a synthetic analogue of ascorbic acid and differs from it only in that it has the opposite optical configuration on carbon atom 5. Isoascorbic acid has only one-twentieth of the antiscorbutic activity of ascorbic acid (19). Therefore, the fact that dehydroisoascorbic acid has less tendency to produce the hyperactive response of dehydroascorbic acid may indicate that the reaction occurring with dehydroascorbic acid is merely an exaggeration of a normal biochemical process.

There is an increased tolerance to a second dose of dehydroascorbic acid with a marked decrease in the characteristic hyperactive reaction that follows the initial dose. The increased tolerance is noted 10 minutes after the initial dose and is prolonged. The fact that a large second dose of dehydroascorbic acid produces less hyperactivity than a smaller initial dose seems to indicate that the hyperactive response is mediated by a substance liberated *in vivo* which is present in a limited quantity. Thus, if all of this substance is liberated at one time, death results, while liberation of most of it causes the characteristic reaction without death. The second dose then merely releases the small amount remaining without causing any effect. It is interesting that some of the effects produced by dehydroascorbic acid are similar to those produced by acetylcholine.

It seems likely that toxic manifestations previously reported for ascorbic acid (20) may have been due to the presence of dehydroascorbic acid.

Dehydroascorbic acid under the conditions used is more effective than ascorbic acid in acting synergistically with alloxan to produce diabetes. It is possible that the effect of ascorbic acid is the result of its conversion to dehydroascorbic acid by the oxyhemoglobin (21) of the blood which is released on hemolysis following the injection of alloxan (22). Dehydroascorbic acid possibly acts by lowering glutathione and thus having a sparing action on alloxan (23).

Permanent hyperglycemia is produced by the injection of 1.4 gm. per kilo of dehydroisoascorbic acid. In a dose of 1 gm. per kilo, a temporary diabetes is produced. In this same dosage dehydroascorbic acid produces essentially the same result as dehydroisoascorbic acid. The general toxicity of dehydroascorbic acid prevents the use of a higher dosage. However, repeated injections of dehydroascorbic acid seem to be capable of producing a permanent diabetes. It is believed that this is the first time that diabetes, seemingly permanent, has been produced by a known chemical substance that is not closely related to alloxan. The fact that dehydroascorbic acid occurs physiologically in man and is capable of producing permanent diabetes is also significant.

There does not appear to be a correlation between the hyperactive response following the intravenous injection of these compounds and their diabetogenic effect. On the basis of the temporary hyperglycemia produced with smaller doses, dehydroascorbic acid and dehydroisoascorbic acid seem to have about the same potency as far as the production of diabetes is concerned. However, dehydroascorbic acid is 4 or more times as effective as dehydroisoascorbic acid in producing a hyperactive response.

The mechanism of action of these substances is probably similar to that of alloxan (13, 24) which is thought to interfere with essential sulfhydryl enzymes of the β cells. The similarity in action to that of alloxan is indicated by the previously demonstrated (25) triphasic glucose response following the injection of dehydroascorbic acid. The action on the β cells with decreased insulin production is indicated by the ready response of the hyperglycemia to injected insulin.

It is too early to generalize about the essential chemical structure required for the production of diabetes, but the results with dehydroascorbic and dehydroisoascorbic acids lead to a greater variation in possible structure. In searching for a precipitating factor in human diabetes, it becomes necessary to broaden the search beyond the nitrogen-containing purines and pyrimidines to substances which may be derived from carbohydrates, and as a matter of fact to any substance that may cause an excessive oxidation of ascorbic acid to dehydroascorbic acid in the β cells of the islets of Langerhans.

SUMMARY

1. Dehydroascorbic acid produces a characteristic reaction in rats with an LD_{50} of about 320 mg. per kilo. Following a sublethal dose, rats will tolerate a second dose 3 to 4 times as large as the initial dose.

2. Dehydroascorbic acid is more effective than ascorbic acid in acting synergistically with alloxan to produce diabetes.

3. Dehydroascorbic acid is shown to be similar to alloxan in structure and chemical properties, and to produce a hyperglycemia of a few days duration following a dose of 1.1 gm. per kilo in rats. Three daily injections of 80 mg. are capable of producing what appears to be permanent diabetes in rats weighing about 120 gm.

4. Dehydroisoascorbic acid produces permanent diabetes in doses of 1.5 gm. per kilo.

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A PARTIAL SEPARATION AND CHARACTERIZATION OF CYTOCHROME OXIDASE AND CYTOCHROME *b**

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Numerous attempts have been made to separate the insoluble cytochromes of mammalian heart muscle. Von Euler and Hellström in 1939 (1) used sodium cholate and ammonium sulfate and obtained a preparation containing only cytochrome *b*. Straub (2) obtained a fraction with sodium cholate and ammonium sulfate in which he demonstrated cytochrome oxidase (called cytochrome a_3 by him after Keilin and Hartree (3)) and very little cytochrome *b*. In the same year Yakushiji and Okunuki (4), also using sodium cholate and ammonium sulfate, claimed the preparation of cytochrome oxidase (called cytochrome *a* by them). To us the preferential solubility in sodium desoxycholate of proteins that are inactive when tested with the hydroquinone-cytochrome *c* system (5, 6) suggested that the various components of the insoluble complex might be separated by successive additions of small amounts of desoxycholate (7).

The characterization of cytochrome oxidase and cytochrome *b* has rested principally on a knowledge of their absorption spectra. Keilin and Hartree (3) found bands at 600 mμ and 448 mμ for reduced cytochrome a_3 (which they suggest may be oxidase), at 605 mμ and 452 mμ for reduced cytochrome *a* (the existence of which we do not consider proved), and at 564, 530, and 432 mμ for reduced cytochrome *b*. Keilin and Hartree (3) and Graubard (8) have suggested that cytochrome oxidase may be a copper-containing enzyme.

This report deals with the details of a partial separation and purification of the cytochromes contained in an insoluble preparation of mammalian heart muscle. Cytochrome *b* and cytochrome oxidase are characterized with respect to their absorption spectra in both the oxidized and reduced

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states. Evidence is presented which indicates that cytochrome oxidase is a copper-containing enzyme.

EXPERIMENTAL

The *insoluble cytochrome complex preparation* was obtained from beef heart and was a suspension precipitated one or three times by acetate as previously described (9, 6).

The *test of enzyme activity* was adapted from the methods of Haas (10), Keilin and Hartree (11), and Stotz, Sidwell, and Hogness (12). The gas phase was air and the temperature 25°. Each vessel contained 3.0 mg. of hydroquinone in 0.25 ml. of water (side arm), 1.0 mg. of cytochrome *c* in 0.25 ml. of water, 0.3 ml. of 0.1 M semicarbazide-HCl (previously adjusted to pH 7.1 with NaOH), 1.45 ml. of 0.1 M Na_2HPO_4-KH_2PO_4 buffer of pH 7.1, 0.25, 0.5, or 0.75 ml. of the enzyme preparation, and distilled water, when necessary, to make the total volume 3.0 ml. The limit of the oxygen uptake by this system is approximately 90 c.mm. in 15 minutes. 1 ml. of the insoluble preparation or 1 "ml. equivalent"[1] of the lyophilized enzyme preparations was dissolved in 25 ml. of cold distilled water for the test.

Copper was determined by the method of McFarlane (13), *iron* by the method of Lintzel (14), and *protein* by the micro-Kjeldahl method by use of the factor 6.25 to convert nitrogen to protein.

Insoluble Complex—The insoluble preparations made from beef heart, when tested manometrically with the hydroquinone-cytochrome *c* system, gave Q_{O_2} protein values at 25° ranging from 260 to 600. A typical preparation, the one from which the preparation next described was prepared, had a Q_{O_2} protein value of 300.

Preparation 0-4—The addition of 40 mg. of sodium desoxycholate[2] (4 per cent) to each ml. of the cold insoluble preparation (precipitated three times by acetate), followed by grinding in a cold mortar, and by centrifugation for 1 hour at 25,000 × *g*,[3] yields a slightly opalescent, red-brown supernatant. The solution may be lyophilized. Approximately 80 per cent of the protein is dissolved, and the Q_{O_2} protein values at 25° range from 1140 to 1840. A preparation made from the insoluble complex discussed in the preceding section had a Q_{O_2} protein of 1840. The observed density values[4] of a solution made by dissolving 1 ml. equivalent in 20 ml. of distilled water were divided by the mg. of protein in the solution. This is justified on the basis of the relationship, $K = D/CL$, where K is a constant, D is the density, C is the concentration of protein (expressed here in mg.), and L is the length of the solution absorbing the light (1 cm. here). The calculated values plotted against the wavelengths are given in Fig. 1. The curve for the oxidized system shows a peak at 414 mμ. The curve for the reduced[5] system has a peak at 428 mμ (cytochrome *b*) with a slight skewing at 415 to 420 mμ (cytochrome *c*) and a marked skewing at about 442 mμ (cytochrome oxidase). The hump with a maximum at 525 mμ and the hump with components at 560 and 550 mμ belong to cytochrome *b* and cytochrome *c*. The 602 mμ peak belongs to cytochrome oxidase. There is evidence, therefore, for the presence of the cytochromes *b*, *c*, and oxidase in this preparation.

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[1] 1 "ml. equivalent" represents the amount of dry preparation obtained by lyophilizing 1 ml. of the concentrated supernatant.

[2] Difco.

[3] International multispeed attachment, six place head, 10 ml. Lusteroid tubes.

[4] Beckman spectrophotometer.

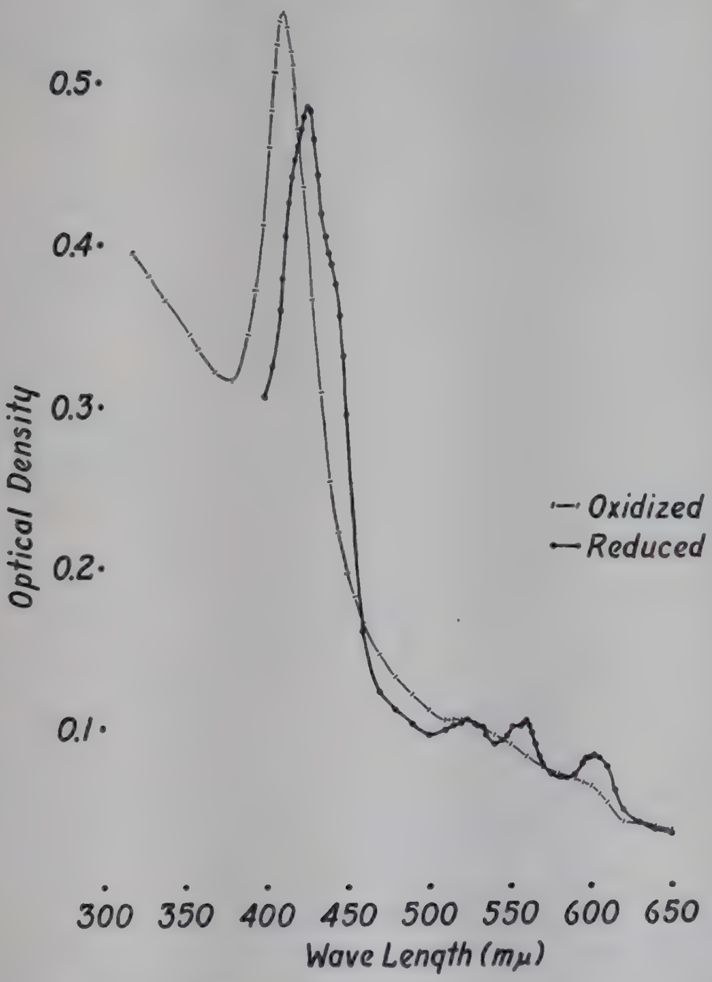

FIG. 1. Preparation 0-4

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[5] Reduced with a pinch of sodium hydrosulfite. The absorption by the reduced enzymes cannot be analyzed below 400 mμ because of the marked absorption by sodium hydrosulfite in this region.

Flavoprotein does not exhibit its 450 mμ peak on the curve of the oxidized system.

Preparations 0-2 and 2-3—The partial purification of cytochrome oxidase, by the use of sodium desoxycholate, has been previously described (6). The same procedure can be made to yield an almost complete separation of the cytochromes *b* and oxidase. If to each ml. of the cold insoluble preparation (precipitated three times by acetate) one adds 20 mg. (2 per cent) of sodium desoxycholate in a cold mortar with grinding or in a beaker with stirring and centrifuges the mixture in the cold at 25,000 × *g* for 1 hour, the supernatant (termed Preparation 0-2) contains mainly cytochrome *c* and cytochrome *b* and only small amounts of cytochrome oxidase. The supernatant can be lyophilized to yield a dry preparation. The Q_{O_2} protein (25°) values of the lyophilized preparations range from 25 to 260; the preparation described here has a value of 230. The absorption spectrum (Fig. 2) of this preparation, made by dissolving 1 ml. equivalent in 10 ml. of cold distilled water is expressed in terms of optical density per mg. of protein ($K = D/CL$), and shows a peak at about 413 mμ in the oxidized state. The curve for the reduced components has a peak at 428 mμ (cytochrome *b*) which is markedly skewed toward the blue by a component absorbing at about 415 to 420 mμ (cytochrome *c*).

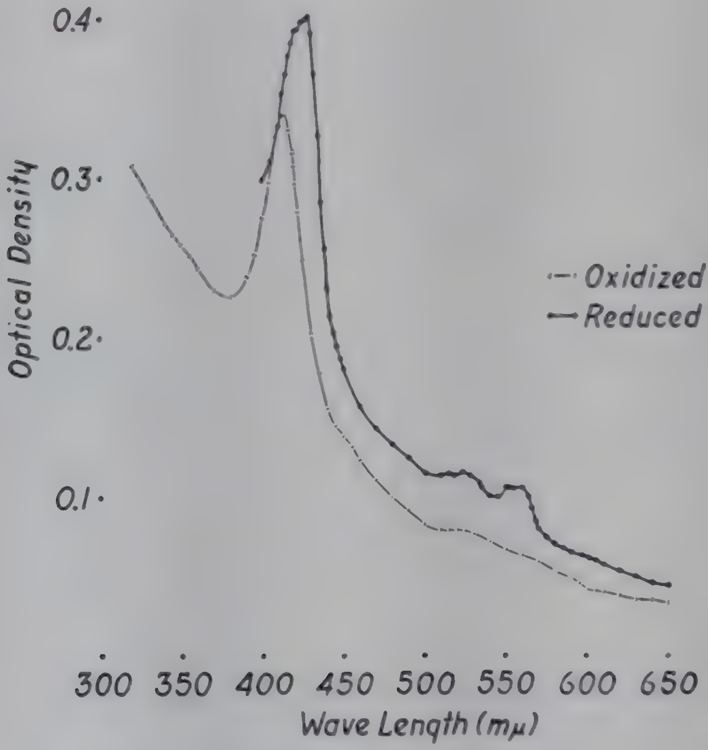

FIG. 2. Preparation 0-2

The hump with a maximum at 522 mμ and that with a plateau at 550 to 560 mμ belong to cytochrome *c* and cytochrome *b*. Sometimes, as in this instance, there is almost no evidence for the presence of cytochrome oxidase (601 mμ peak in the reduced state) or of flavoprotein (450 mμ peak in the oxidized state). More commonly, however, a small 601 mμ peak is present.

The precipitate from Preparation 0-2 may be further treated with sodium desoxycholate. The precipitate is taken up in 0.1 M Na_2HPO_4-KH_2PO_4 buffer of pH 7.4 and reduced to a uniform suspension in a glass tissue homogenizer. More buffer is added until the final volume is equal to the volume of the insoluble preparation first taken for Preparation 0-2. To each ml. of the suspension there are added 30 mg. (3 per cent) of sodium desoxycholate in a cold mortar with grinding or in a cold beaker with stirring and the mixture is centrifuged in the cold for 1 hour at 25,000 × *g*. The supernatant (Preparation 2-3) can then be lyophilized. The Q_{O_2} protein (25°) values of the dry preparations range from 2080 to 2880. The one described here had a Q_{O_2} protein of 2880. The absorption curves of a solution, made by dissolving 1 ml. equivalent in 5 ml. of cold distilled water is expressed in terms of optical density per mg. of protein ($K = D/CL$), and indicates the presence principally of cytochrome oxidase (Fig. 3). The curve of the oxidized enzymes has a peak at about 416 mμ (cyto-

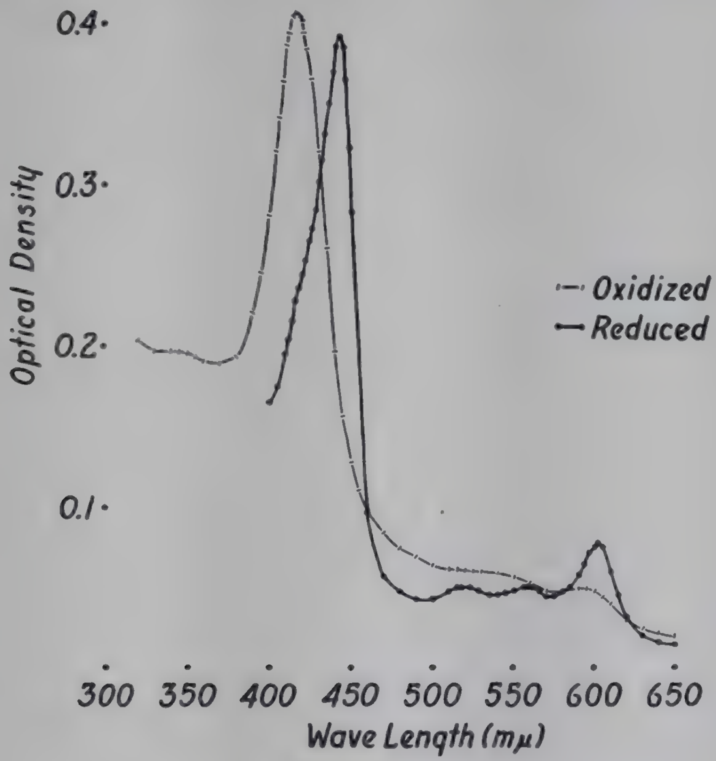

Fig. 3. Preparation 2-3

chrome oxidase) and another at 590 to 595 mμ (cytochrome oxidase). The curve of the reduced enzymes has a peak at 442 mμ (cytochrome oxidase), a hump with a maximum at about 523 mμ (cytochrome *b*), another hump with a maximum at 560 mμ (cytochrome *b*), and a peak at 602 mμ (cytochrome oxidase).

Series Fractionation—It was previously demonstrated (6) that the addition of 1 per cent of sodium desoxycholate to an insoluble preparation would dissolve approximately 25 per cent of the proteins and that the solution would exhibit little or no oxidase activity. This fact and a study of the absorption curves for Preparations 0-2 and 2-3 suggested the successive addition of 1 per cent of desoxycholate to separate the constituent enzymes. An insoluble preparation, precipitated three times by acetate, is used rather than a preparation precipitated only once, because it is assumed that the former is less contaminated by hemoglobin. Each ml. of the insoluble preparation is treated with 10 mg. of sodium desoxycholate in a cold beaker with stirring and then centrifuged in the cold at 25,000 × *g* for 1 hour. The supernatant, which is usually somewhat opalescent, is poured off and termed Fraction A. The precipitate is taken up in 0.1 M Na_2HPO_4-KH_2PO_4 and reduced to a uniform suspension in a glass tissue homogenizer. More buffer is added until the volume of the suspension is equal to the volume of the insoluble preparation first taken. To each ml. of the suspension are added 10 mg. of sodium desoxycholate in a cold beaker with stirring. Following centrifugation, as before, the supernatant, termed Fraction B, is poured off and lyophilized. The precipitate is further treated as just described until five fractions have been obtained. The preparation is sometimes complicated by the formation of a gel when either the fourth or the fifth fraction is being stirred in the beaker. Warming the beaker in the hand, accompanied by slow stirring, followed by rapid pipetting into the centrifuge tubes, usually suffices to delay the gel formation. However, if gelation is suspected, it is best to stop the centrifuge at the end of ½ hour and to inspect the tubes. If a gel layer is present, the tubes should be warmed in the hand, the contents stirred slowly until the gel is dispersed, and promptly placed in the centrifuge.

For spectral analysis Fractions A and B were so prepared that each ml. equivalent was dissolved in 2 ml. of distilled water. Fractions C, D, and E were made so that each ml. equivalent was dissolved in 1 ml. of distilled water. The observed density values were divided by the mg. of protein in the preparation ($K = D/CL$) and plotted against the wavelength. The curves for Fraction A (Q_{O_2} protein (25°) 87) are presented in Fig. 4. The curve for the oxidized preparation has a peak at 410 mμ. The curve for the reduced preparation has a peak at 420 mμ, a hump with a maximum at 548 mμ, and another small peak at 595 mμ. This fraction, which is always opalescent and, therefore, has an uncertain baseline, gives indications of containing small amounts of cytochromes *c*, *b*, and oxidase.

Fraction B (Q_{O_2} protein 1430) has a peak at 414 mμ (cytochrome *b*) in the oxidized state (Fig. 5) which is skewed toward 408 mμ (cytochrome *c*). In the reduced state there is a peak at 428 mμ (cytochrome *b*) which is unsymmetrical on the left and gives indications of a component at 415 mμ (cytochrome *c*). The hump with a plateau at 525 to 530 mμ and the peak at 560 mμ which is slightly skewed toward 550 mμ indicate the presence of cytochrome *b* and of a little cytochrome *c*. The presence of cytochrome oxidase is indicated by the plateau at 599 to 605 mμ.

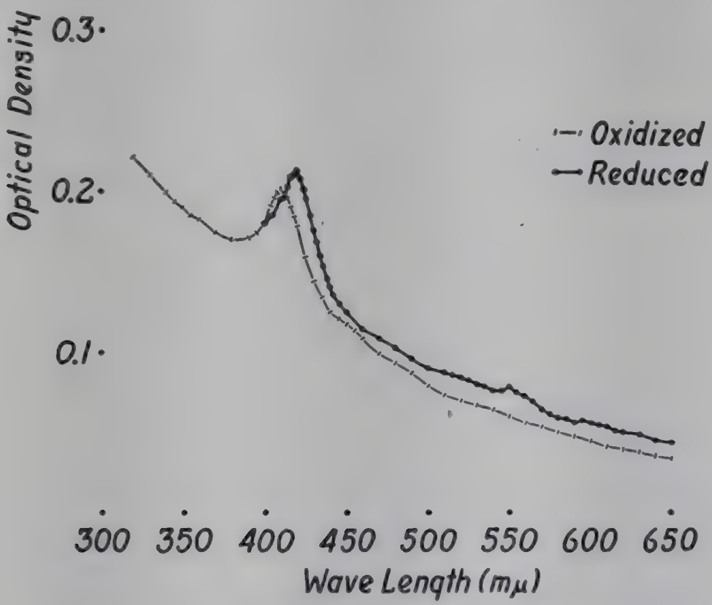

FIG. 4. Fraction A

Fraction C (Q_{O_2} protein 1840) has a peak on the curve for the oxidized system at 416 mμ (Fig. 6). In the reduced state there is a peak at 428 mμ (cytochrome *b*), with an accompanying component absorbing strongly at about 440 mμ (cytochrome oxidase). There are peaks at 528 mμ (cytochrome *b*), 558 mμ (cytochrome *b*), and 600 mμ (cytochrome oxidase).

Fraction D (Q_{O_2} protein 650) in the oxidized state (Fig. 7) has a peak at 418 mμ and a small hump with a plateau at 590 to 595 mμ, both of which belong to cytochrome oxidase. The peak at 440 mμ in the reduced state belongs to cytochrome oxidase. It is markedly skewed toward the blue by a component absorbing at about 430 mμ (cytochrome *b*). The hump with a plateau at 510 to 530 mμ and the 560 mμ peak belong to cytochrome *b*. The 601 mμ peak belongs to cytochrome oxidase. Fraction E can be shown to have cytochromes *b* and oxidase, although in lesser amounts. The curves for Fraction E are not given because they are very irregular after having been expressed in terms of optical density per mg. of protein ($K = D/CL$). The protein content is low and all density readings are, therefore, multiplied by a large conversion factor.

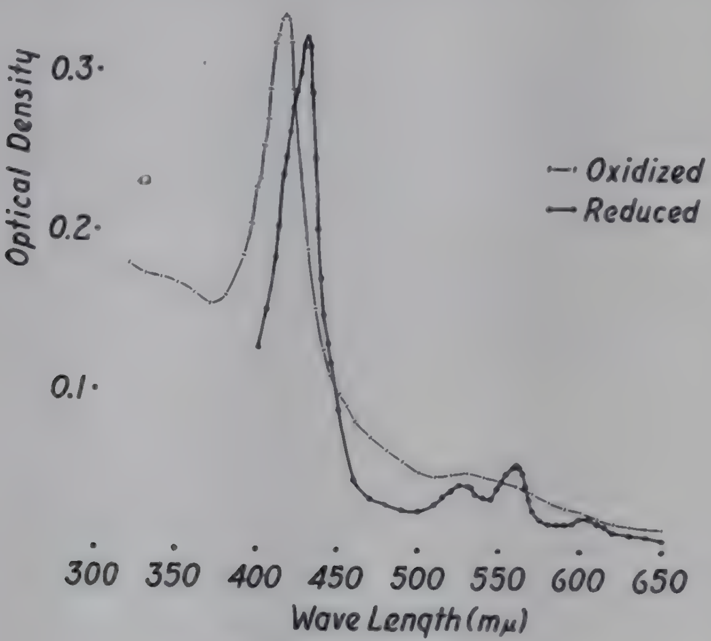

FIG. 5. Fraction B

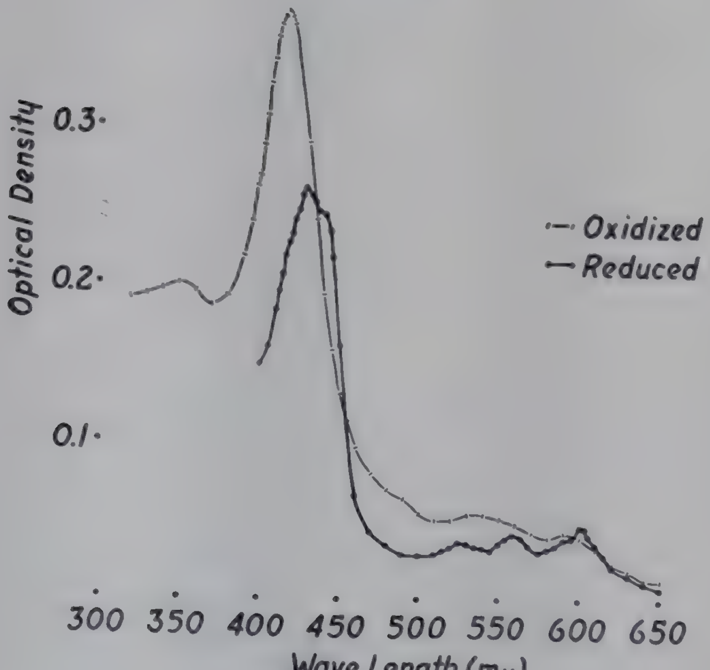

FIG. 6. Fraction C

A summary of the absorption values obtained following fractionation is given in Table I where the values for cytochrome *b* and cytochrome oxidase are presented. Cytochrome *b* in the oxidized state has a prominent peak at 414 mμ. In the reduced state it has peaks at 428, 525, and 560 mμ. The relative heights of these three peaks (the 428 mμ peak being

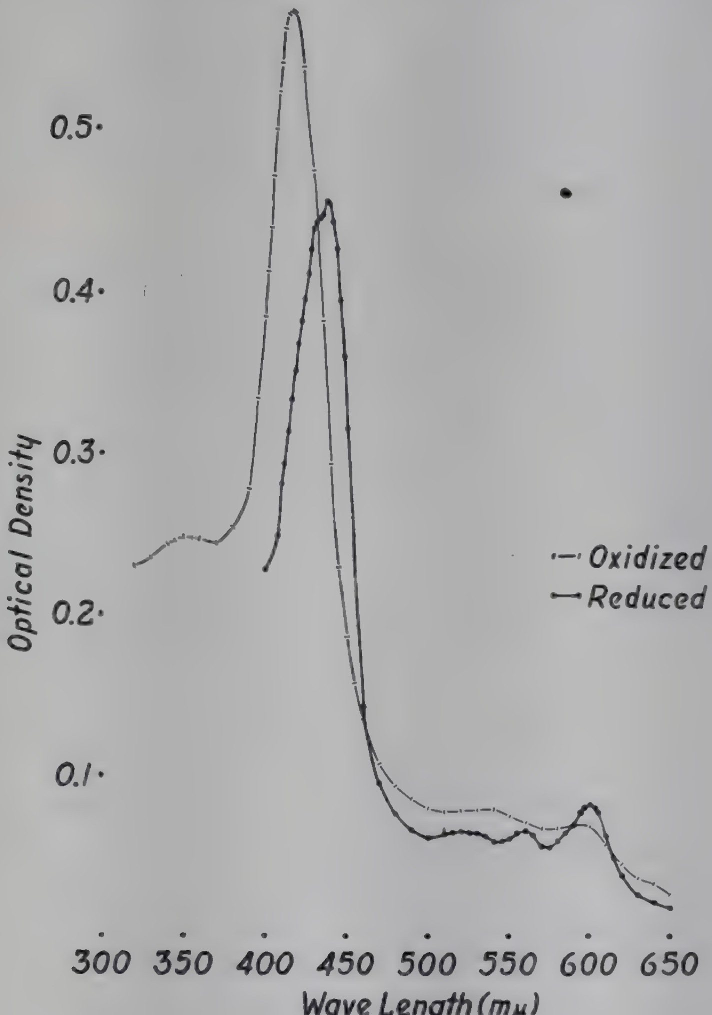

FIG. 7. Fraction D

TABLE I

Absorption Maxima

Preparations	Cytochrome *b*				Cytochrome oxidase*			
	Oxidized	Reduced			Oxidized		Reduced	
	mμ	mμ	mμ	mμ	mμ	mμ	mμ	mμ
This paper	414	428	525	560	418	590–595	440	601
Keilin and Hartree (3)		432	530	564			448	600

* Keilin and Hartree's cytochrome a_3.

very prominent) and the position of the peaks (compare the values for cytochrome *c*, 415, 520, and 550 mμ) strongly suggest that cytochrome *b* is an iron-porphyrin enzyme. Our values for reduced cytochrome *b* are consistently lower than the values reported by Keilin and Hartree (3) (see Table I). We have, however, repeatedly obtained the same values with several spectrophotometers for either beef or hog heart. Furthermore, we have always checked our instruments against cytochrome *c* prepared from beef heart and have obtained a characteristic curve for the reduced compound.

Oxidized cytochrome oxidase has a high, sharp peak at 418 mμ and a low, flat peak at 590 to 595 mμ. In the reduced state it absorbs markedly at 440 mμ and at 601 mμ. Our 440 mμ value does not agree with that of 448 mμ given by Keilin and Hartree. The fact that there are only two peaks for cytochrome oxidase in the reduced state and three peaks for cytochrome *c* and cytochrome *b* suggests that cytochrome oxidase differs from these compounds.

Copper and Iron Analyses—A large amount of an insoluble heart muscle suspension (approximately 200 ml.), precipitated once by acetate, was made from beef heart by use of buffers that had been prepared with water redistilled from Pyrex glass. The buffer salts were of reagent grade and were found to be practically free of copper and iron. Preparations 0-4, 0-2, and 2-3 and Fractions A, B, C, and D were prepared from this insoluble suspension and lyophilized for use. The sodium desoxycholate was precipitated as desoxycholic acid five times by the addition of H_2SO_4 (redissolved each time with NaOH) and finally adjusted to its initial pH and lyophilized to yield a preparation free of copper and very low in iron. All glassware was carefully washed with water that had been redistilled from Pyrex glass. The values obtained for protein, oxygen uptake, copper, and iron, and for the height of the 601 mμ peak on the absorption curve of the reduced preparation are given in Table II. A discussion of the values in each column follows.

Protein—4 per cent of desoxycholate dissolves about 80 per cent of the proteins contained in the insoluble preparation. The sum of the proteins dissolved by 2 and 3 per cent of desoxycholate added successively (Preparation 0-2 + 2-3) is about 95 per cent of the proteins contained in the insoluble preparation.

15 Minute Oxygen Uptake—In the test for activity all the enzyme solutions were used in the dilute (1:25) form. 0.25 ml. of Preparations 0-4 and 2-3, 0.5 ml. of the insoluble preparation and Preparation 0-2, and 0.75 ml. of Fractions A through D were used.

1 Hour Oxygen Uptake—This is a calculated value. Column 2 is multiplied by 4 to increase the time to 1 hour, and multiplied by 25 to account for the dilution.

TABLE II

Analyses of Various Preparations

Preparation No.	Protein* (1)	15 min. oxygen uptake† (2)	1 hr. oxygen uptake* (3)	Q_{O_2} protein (25°) (3) ÷ (1) (4)	Copper in sample analyzed (5)	Copper per ml.* (6)	Copper-protein ratio, (6) ÷ (1) (7)	Iron in sample analyzed (8)	Iron per ml.* (9)	Iron-protein ratio (9) ÷ (1) (10)	Height of 601 mμ peak* (11)	Copper-601 mμ ratio (6) ÷ (1) (12)	Iron-601 mμ ratio (9) ÷ (11) (13)	Oxygen uptake-601 mμ ratio (3) ÷ (11) (14)
	mg.	*c.mm.*	*c.mm.*	*c.mm. per mg. per hr.*	γ	γ	*γ per mg.*	γ	γ	*γ per mg.*	*density units*	*γ per density unit*	*γ per density unit*	*c.mm. per density unit*
Insoluble	22.45	58	5,800	260	29.6	4.9	0.22	12.1	12.1	0.54				
0-4	17.95	232	23,200	1290	15.0	3.0	0.17	8.8	8.8	0.49	1.180‡			
0-2	16.25	12	1,200	70	5.4	1.1	0.07	20.1	6.7	0.41	0.910‡			
2-3	5.05	112	11,200	2220	11.0	2.2	0.44	7.3	2.4	0.47	0.460	4.8	5.2	24,400
A	5.78	7	700	120	5.6	0.56	0.10	15.3	3.8	0.66	0.340‡			
B	2.38	35	3,500	1470	5.8	0.58	0.24	4.0	1.3	0.55	0.082	7.1	15.9	42,700
C	0.98	17	1,700	1740	5.8	0.58	0.59	0.9	0.2	0.20	0.092	6.3	2.2	18,500
D	1.08	7	700	650	9.1	0.91	0.84	<0.6	<0.2	<0.19	0.147	6.2	<1.4	4,800

* Per ml. of concentrated supernatant.

† Per ml. of dilute supernatant (1:25).

‡ Value doubtful; see the text for explanation.

Q_{O_2} *Protein (25°)*—These values are fairly typical. The low value for Fraction D, which contains cytochrome oxidase on the basis of its absorption spectra, is difficult to explain. The discrepancy may be due to at least one of the following: (*a*) an inhibition by desoxycholate of the oxidation of reduced cytochrome *c* by oxidase in the test for activity (observed both manometrically and spectrophotometrically); (*b*) denaturation of the cytochrome oxidase during the preparation; (*c*) a separation of factors not yet recognized.

Copper in Sample Analyzed—5 ml. or 5 ml. equivalents were used in analyzing the insoluble preparation and Preparations 0-4, 0-2, and 2-3. 10 ml. equivalents of Fractions A through D were used. The results are considered to be correct to within ±10 per cent. The copper contained in Preparations 0-4, 0-2, and 2-3 is non-dialyzable. Samples were dialyzed with slow stirring against 6 liters of distilled water at 4° for 4 days. The copper content and the ratio of copper to protein in each sample was unchanged.

Copper Per Ml.—It is to be noted that the copper content of Fraction D is the highest of all four fractions and that Fraction D always exhibits the most prominent 601 mμ peak.

Copper-Protein Ratio—The value for Preparation 0-2 is low and the value for Preparation 2-3 is high when compared to the insoluble preparation. It is to be noted that Preparation 2-3 had a prominent peak at 601 mμ, while Preparation 0-2 had none. See Figs. 2 and 3 for curves that are almost exact replicas of the curves for these preparations.

Iron in Sample Analyzed—1 ml., and 1 ml. equivalent of the lyophilized preparation, respectively, were used in analyzing the insoluble preparation and Preparation 0-4. 3 ml. equivalents were taken of Preparations 0-2 and 2-3 and of Fractions B and D, and 4 ml. equivalents of Fractions A and C. The results are considered to be correct to within ±10 per cent. The iron contained in Preparations 0-4, 0-2, and 2-3 is non-dialyzable. Samples were dialyzed with slow stirring against 6 liters of distilled water at 4° for 4 days. The iron content and the iron-protein ratios were unchanged.

Iron Per Ml.—It is to be noted that the iron content decreases markedly in Fractions A to D. The value for Fraction D is essentially zero.

Iron-Protein Ratio—The ratios are essentially constant for all preparations except that Fraction C has a low ratio and Fraction D has a ratio somewhere near zero.

Height of 601 mμ Peak—These density values were obtained with the spectrophotometer. The values for Preparations 0-4 and 0-2 and for Fraction A are not considered significant because the opalescence (probably due to fat) raised the base-line to an unknown height above zero.

Ratio of Copper to 601 mμ Peak—The ratios are fairly constant. The correlation is considered good for Preparation 2-3 and for Fractions B, C, and D. These constant ratios are considered to indicate that copper is associated with the cytochrome component that absorbs light at 601 mμ.

Ratio of Iron to 601 mμ Peak—These ratios are not constant. They range from 15.9 for Fraction B to near zero for Fraction D. The poor correlation is interpreted to mean that iron is not contained in the component that absorbs at 601 mμ.

Ratio of Oxygen Uptake to 601 mμ Peak—The ratios are not constant, ranging from 4800 to 42,700. Unless the ratios are inaccurate for the reasons presented above under "Q_{O_2} protein (25°)," this would indicate that the oxygen uptake is not proportional to the concentration of the component which absorbs at 601 mμ. However, the preparations that have a prominent 601 mμ peak, with the exception of Fraction D (see Figs. 1, 3, 5, and 6), also have a high Q_{O_2} protein.

DISCUSSION

A comparison of the absorption spectra of our Preparation 0-4 and of Straub's preparation (2), which he made with sodium cholate, ammonium sulfate, sodium hydrosulfite, and heat, reveals a marked similarity. However, Straub reports that another of his preparations, which is essentially free of cytochrome *b* and which has an absorption curve very much like our Preparation 2-3, has a Q_{O_2} protein of 300 at 38°. This is to be contrasted to the Q_{O_2} protein of 2880 at 25° for our Preparation 2-3.

Keilin and Hartree (3) have reported the presence of two cytochrome *a* components, namely, cytochromes *a* and a_3. According to these authors the two enzymes have the same absorption spectra and can be differentiated best by forming the carbon monoxide compound of the reduced cytochrome a_3. Straub arrives at the same conclusion, although the peak that he attributes to cytochrome *a* is more probably the 428 mμ peak of cytochrome *b*. We have adopted the term oxidase for the compound which absorbs strongly at 442 and 601 mμ, and we have compared its absorption peaks with Keilin and Hartree's cytochrome a_3. Keilin and Hartree suggest that if cytochrome a_3 has a third band in the visible region it is masked in their preparations by the 550 and 560 mμ bands of the cytochromes *c* and *b*. We have never encountered a third peak for cytochrome oxidase. This enzyme, which absorbs at 442 mμ and at 601 mμ, appears to be the one that oxidizes hydroquinone in the presence of cytochrome *c*, oxidizes reduced cytochrome *c*, and is inhibited by carbon monoxide, cyanide, and azide (6). Although the correlation between the oxygen uptake catalyzed by a preparation and the height of the peak at 601 mμ is not always good, in general those preparations that have a prominent peak at 601 mμ also have a high Q_{O_2} protein. The poor correlation may be due in part to the fact that sodium desoxycholate interferes with the oxidation of reduced cytochrome *c* by cytochrome oxidase in the test of activity.

There is a marked difference in color between Preparations 0-2 and 2-3. A solution of Preparation 0-2, although somewhat opalescent, is red in color and highly suggestive of a solution of cytochrome *c*. In view of the spectrophotometric observation that this preparation contains cytochrome *b* with but small amounts of cytochrome *c*, it appears likely that cytochrome *b* is an iron-porphyrin compound. In marked contrast to the color of Preparation 0-2 is the yellow-green of Preparation 2-3, especially when it can be demonstrated spectrophotometrically that the latter contains very little cytochrome *b*. Since many copper compounds are green in color, the suggestion that cytochrome oxidase is a copper-containing enzyme is strengthened.

Both Keilin and Hartree (3) and Graubard (8) have suggested that cytochrome oxidase might be a copper-containing enzyme. The conclusion of Keilin and Hartree is based on such observations as the universal distribution of intracellular copper, the facility with which copper salts oxidize reduced cytochrome *c*, the work of Cohen and Elvehjem discussed below, and a certain similarity in properties between cytochrome oxidase and polyphenol oxidase, which is a copper-protein compound. Graubard, more directly, inhibited the oxidation by cytochrome oxidase of *p*-phenylenediamine by adding certain reagents that are known to combine with copper. His experiments were in accord with the earlier experiments of Cohen and Elvehjem (15) and Schultze (16, 17). Cohen and Elvehjem found a decreased oxidase activity in the livers of rats made anemic on a milk diet. The feeding of copper increased the oxidase activity, while the feeding of iron did not. Schultze found the oxidase activity of rat liver, heart, and bone marrow to be greatly reduced on a copper-deficient diet. He as well as Cohen and Elvehjem concluded that copper was essential for the formation and maintenance of cytochrome oxidase activity. Our experiments offer direct evidence from analyses and indirect evidence from comparison with the absorption spectrum of reduced cytochrome *c* that cytochrome oxidase is a copper-containing enzyme and not an iron-containing enzyme.

SUMMARY

1. Cytochrome oxidase and cytochrome *b* of mammalian heart muscle have been almost completely separated by the use of sodium desoxycholate.

2. Cytochrome oxidase in the oxidized state (with sodium desoxycholate present) has a high, sharp peak in its absorption curve at 418 mμ and a very low, flat peak at 590 to 595 mμ. In the reduced state it absorbs

strongly at 440 and 601 mμ. The absence of a third peak, the presence of which is so characteristic of cytochrome *c*, suggests that cytochrome oxidase differs in its composition from this compound.

3. Cytochrome *b* in the oxidized state (with sodium desoxycholate present) displays a prominent absorption peak at 414 mμ. In the reduced state it absorbs strongly at 428, 525, and 560 mμ. A consideration of the general contour of the curves, the relative heights of the peaks, and the position of the peaks when compared with the same for cytochrome *c* suggests that cytochrome *b* may be an iron-porphyrin enzyme.

4. The good correlation obtained with several fractions between the copper content and the height of the 601 mμ peak in the reduced curve is considered to indicate that cytochrome oxidase is a copper-containing enzyme. This conclusion is strengthened by the observation that the correlation between the iron content and the height of the 601 mμ peak is poor.

5. The lack of correlation in many instances between the oxygen uptake catalyzed by a preparation and the absolute height of the absorption peak at 601 mμ remains unexplained. In general, however, the preparations that have a prominent 601 mμ peak in the reduced state also have a high Q_{O_2} protein.

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PYRIMIDINE NUCLEOSIDES AS PRECURSORS OF PYRIMIDINES IN POLYNUCLEOTIDES

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The purines and pyrimidines, so far the only known nitrogen-containing components of the polynucleotides, differ, with the exception of adenine, from most other nitrogenous substances, *e.g.* amino acids, in that when administered with the food they are not utilized by the rat. This was first shown by Plentl and Schoenheimer (1), who fed N^{15}-labeled guanine, uracil, and thymine to rats and isolated the bases from the polynucleotides. They showed that in no case had isotope entered the purines or pyrimidines. The only substances containing the isotope in a significant amount were allantoin, after feeding guanine, and urea and ammonia after the pyrimidines were fed. These products were isolated from urine. The same negative results were obtained by Bendich, Getler, and Brown (2) with cytosine as the isotopic precursor.

From these experiments the conclusion might be drawn that the pyrimidines present in food in the form of polynucleotides cannot act as precursors for the synthesis of new polynucleotides by the rat. This does not necessarily follow if, as is generally assumed, the digestive enzymes do not split off the free bases from dietary polynucleotides. Nucleosides formed by partial hydrolysis may be absorbed through the intestinal wall and these, unlike the free pyrimidines, might then be utilized for the synthesis of new polynucleotides by the animal.

That a difference exists between free pyrimidines and pyrimidine nucleosides or nucleotides for certain organisms has been shown by Loring and Pierce (3) in experiments on mutants of *Neurospora* which require uracil for growth. Cytosine did not permit any growth. Both uridine and cytidine or the corresponding nucleotides could, however, be used instead of uracil. Furthermore the nucleosides and nucleotides allowed 10 to 60 times more growth than did an equimolar amount of uracil. The much higher activity of the nucleosides compared with that of the free bases may indicate a fundamental difference between the metabolism of free pyrimidine and pyrimidine bound to ribose. This difference may be valid for the rat also.

In order to investigate this question, cytidine and uridine, labeled in the rings and the amino group with N^{15}, have been injected into rats. A preliminary report of these experiments has been published previously (4). At about the same time Brown *et al.* (5) reported the results of experiments in which a biologically (N^{15}) marked hydrolysate of yeast nucleic acid was administered to rats. The isolated mixed pyrimidines contained a significant amount of the isotope, showing that the hydrolysate served as a precursor for the pyrimidines in polynucleotides.

In the present investigation the N^{15} nucleosides were prepared by growing yeast on a medium containing ammonia with an excess of N^{15} as the only source of nitrogen. Pyrimidine nucleosides were isolated from the ribose nucleic acid (PNA) of this yeast according to methods worked out in this laboratory (6–8).

TABLE I

Injection of Cytidine-N^{15} into Rats (Total of 25 Mg. per 100 Gm. of Body Weight during 3 Days)

For the value $E_{max.}$/(microgram of N per ml.) see Reichard (7, 14) and Edman *et al.* (9).

		N^{15} atom per cent excess	$\frac{E_{max.}}{\gamma \text{ N per ml.}}$	Per cent N^{15} calculated on basis of 100 per cent N^{15} in nucleoside injected
	Injected, cytidine..........	6.93	0.297	100
Isolated	PNA			
	Cytidine	0.463	0.303	7.20
	Uridine	0.385	0.321	5.94
	Adenine	0.003	0.178	0.05
	Guanine	0.005	0.162	0.08
	DNA			
	Cytosine	0.281	0.228	4.37
	Thymine	0.101	0.275	1.57
	Adenine	0.074	0.169	1.15
	Guanine	0.028	0.167	0.44
	"Protein"	0.006		0.10

Two rats each received subcutaneously a total of 25 mg. of one of the nucleosides per 100 gm. of body weight, divided into six injections at twelve hourly intervals. The rats were killed 12 hours after the last injection and the polynucleotides were isolated from the pooled internal organs. The PNA and the desoxyribose nucleic acid (DNA) were isolated (6) and the bases and nucleosides, respectively, were prepared from each fraction (7–9, 14) and analyzed for N^{15}.

The results from the *cytidine* injection (Table I) clearly indicate that this nucleoside was a good precursor not only for the pyrimidines of PNA but also for those of DNA. As might be expected, the cytosine in both cases had a higher isotope content than the other pyrimidine. The purines from PNA did not contain a significant amount of N^{15} and the same was true of the trichloroacetic acid-insoluble or "protein" fraction. In the case of the DNA the purines, especially adenine, contained a significant amount of the isotope.

TABLE II

Injection of Uridine-N^{15} *into Rats (Total of 25 Mg. per 100 Gm. of Body Weight during 3 Days)*

		N^{15} atom per cent excess	$\frac{E_{max.}}{\gamma \text{ N per ml.}}$	Per cent N^{15} calculated on basis of 100 per cent N^{15} in nucleoside injected
	Injected, uridine............	11.20	0.325	100
Isolated	PNA			
	Cytidine	0.053	0.298	0.47
	Uridine	0.047	0.325	0.42
	Adenine	0.010	0.179	0.09
	Guanine	0.009	0.166	0.08
	DNA			
	Cytosine	0.055	0.218	0.49
	Thymine	0.039	0.269	0.34
	Adenine	0.033	0.181	0.29
	Guanine	0.049	0.159	0.44
	"Protein"	0.012		0.11

When *uridine-*N^{15} was injected, the isotope entered the different nitrogen-containing substances of both PNA and DNA in rather small amounts (Table II), the pyrimidines showing only a slightly higher isotope content than the purines.

DISCUSSION

Cytidine As Precursor—The present results show that cytidine, administered in small amounts, can be utilized by the rat for the synthesis of new polynucleotides. The isotope in the cytosine of the PNA is so little diluted compared with the administered cytidine that the isotope content in the pyrimidines of the polynucleotides cannot be due to the isotopic amino group of the administered cytidine. This is especially true if one considers the low isotope content in purines from PNA and proteins. It is obvious that the pyrimidine ring of cytidine must have entered the different pyrimidines in the PNA and DNA.

A very interesting finding is the rather high amount of isotope in the pyrimidines of the DNA. The turnover ratio PNA:DNA is about 2:1 for cytosine, a finding which agrees well with the results obtained by Hammarsten and Hevesy (10) for the phosphorus of the whole rat and is in marked contrast to the results obtained by Brown *et al.* (11) with adenine-N^{15} as precursor for purines from polynucleotides (29:1).

The occurrence of the isotope in the DNA pyrimidines suggests that the rat is able to form desoxyribose from ribose when the sugar is bound to cytosine. This agrees with the conclusion of Bendich, Getler, and Brown (2) that free cytosine cannot be utilized for the synthesis of DNA pyrimidines. Thus cytosine cannot be split off from ribose and then linked to desoxyribose for the synthesis of the DNA. This is in agreement with the results obtained by Bredereck *et al.* (12) concerning the point of attachment of desoxyribose to the pyrimidines. These authors found by methylation experiments that the sugar was attached in position 3 of cytosine and thymine. According to our hypothesis, the attachment of desoxyribose to the pyrimidines in DNA is the same as that of the ribose to the pyrimidines in PNA. The latter has been firmly established to be in position 3.

The relatively high content of the isotope in the purines of the DNA, especially in adenine, was unexpected. This cannot very well be ascribed to the amino group of the administered N^{15}-cytidine because the purines from the PNA contain much less of the isotope. The data may possibly indicate some connection between the turnover of purines of the DNA and pyrimidines. This point, however, needs further confirmation.

Uridine As Precursor—When the same amount of uridine-N^{15} was injected into the rat, the results were quite different from those obtained with cytidine-N^{15}. The dilution of the isotope in the PNA pyrimidines was about 15 times higher than when cytidine was used. Furthermore, the isotope, especially in the case of DNA, was distributed much more evenly between the pyrimidines and the purines. It would seem therefore that the utilization of uridine for the synthesis of polynucleotide pyrimidines is very low and is not very specific.

The present experiments also give some evidence concerning the reactions involved in the utilization of orotic acid for the synthesis of the pyrimidines. It has been shown that orotic acid may act as a precursor for pyrimidines in both PNA and DNA (13, 14), but it is not clear how this compound is transformed into uridine and cytidine. Previous experiments on *Neurospora* (3) indicated that the first step might involve a transformation of the orotic acid riboside to uridine. This was disproved, however, by experiments in which the distribution of the isotope between uridine and deaminated cytidine (14) led to the conclusion that uridine and cytidine were formed from orotic acid independently of each other. The present findings and the previous experimental data suggest that orotic acid after amination is first transformed to cytidine and that uridine is then formed from this cytidine.

On the other hand, this general concept of the formation of uridine via cytidine is contradictory to the results of studies of the turnover of PNA pyrimidines in rats after the injection of glycine-N^{15} (14). In these experiments in which the ribosides were prepared from the PNA of different organs, the turnover of the cytosine ring was generally lower than that of uridine. In this case uridine did not appear to have been formed from cytidine.

A similar situation has been found previously in respect to the synthesis of polynucleotide guanine from adenine. This biosynthesis has been proved by the experiments of Brown *et al.* (15) with adenine-N^{15} as precursor. When glycine-N^{15} was used as precursor for polynucleotide purines, however, this synthetic route was not found in all the organs investigated (14). It is concluded that *one* synthetic route does not exist for pyrimidines and purines, but that these substances may be built up in different ways and from different precursors.

SUMMARY

Biologically marked cytidine and uridine have been isolated from yeast grown on a medium containing ammonia with an excess of N^{15} as the source of nitrogen.

The results of the separate injection of the nucleosides into rats show that cytidine is a precursor of PNA and DNA pyrimidines.

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THE INHIBITION OF THE SUCCINOXIDASE SYSTEM BY α-TOCOPHERYL PHOSPHATE AND SODIUM DODECYL SULFATE*

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In attempts to gain information on the biological function of α-tocopherol, a number of investigations have been made dealing with the inhibitory effect of α-tocopheryl phosphate (α-TPh) on enzyme systems. The results obtained with the phosphate ester have served as a basis for speculations on the biological function of vitamin E and have led to the suggestion that the vitamin functions to control the rate of biological oxidations (1), to prevent the breakdown of diphosphopyridine nucleotide (DPN) (2), and to control intravascular blood clotting (3). The principal studies have been concerned with the inhibition of the succinoxidase system by α-TPh. These and other related studies have been recently briefly reviewed (4). In addition, α-TPh has been shown to inhibit trypsin (3), liver acid phosphatase (5), lactic dehydrogenase (2), and DPN nucleosidase (6).

The experiments reported herein were undertaken to ascertain whether the varied effects of α-TPh might be related to the properties of tocopheryl phosphate as an anion with a large non-polar group. Such anions are known to act as protein denaturants and enzyme inhibitors (7). In addition, experiments are reported which give a further understanding of the mechanism by which dodecyl sulfate and α-TPh inhibit the succinoxidase system.

Methods

The manometric succinoxidase[1] assay procedure employed was essentially that of Schneider and Potter (8) for rat liver homogenates with the use of twice recrystallized sodium succinate as substrate. Solutions of the inhibitors, the disodium salt of *dl*-α-tocopheryl phosphate[2] (α-TPh) or sodium dodecyl sulfate[3] (SDS), were added at the expense of water. The

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[1] The term succinoxidase refers to the complete system, including, besides succinic dehydrogenase, the cytochrome components enabling reaction with molecular oxygen.

[2] Kindly supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey.

[3] A purified preparation kindly supplied by E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

cytochrome *c* was prepared according to Potter's modification (9) of the method of Keilin and Hartree (10). Trials with different levels of cytochrome *c* were made to insure that cytochrome was not the limiting component.

In the cytochrome oxidase studies two tissue concentrations were used. The higher level of tissue was the same as in dehydrogenase studies with methylene blue and was used to make the observations more directly comparable as to the amount of tissue and inhibitor used. Since the amount of cytochrome *c* was not sufficient to saturate the cytochrome oxidase system, other experiments were performed with a lower tissue concentration in which the cytochrome *c* concentration was sufficient for saturation. The addition of Ca^{++} and Al^{+++} as activators (8) was not made in the studies with heart homogenate because the cations form insoluble salts with α-TPh. These cations have not been proved to be essential for the activity of the succinoxidase system. The activation seems to be related to the colloidal phosphates which these cations form with the phosphate buffer (11).

For the cytochrome oxidase assay, ascorbate was used to maintain the cytochrome *c* in the reduced form, as has been done by previous investigators (8). The flasks with the higher tissue level contained 0.50 ml. of 0.21 M phosphate buffer, pH 7.4, 0.50 ml. of 0.50 M sodium succinate, 0.80 ml. of 1.8×10^{-4} M cytochrome *c*, 0.50 ml. of 5 per cent rat heart homogenate, and sufficient water to make a total volume of 3.4 ml. The flasks with lower tissue level contained 0.50 ml. of phosphate buffer, 0.30 ml. of sodium succinate, and 1.3 ml. of cytochrome *c*, of the same corresponding concentrations as for the higher tissue level, together with 0.5 ml. of a 1 per cent rat heart homogenate and water to make a total volume of 3.4 ml. The side arms in all cases contained 0.4 ml. of 0.11 M sodium ascorbate, pH 7.0, which had been prepared immediately before using.

Readings with succinate as the only substrate were taken during the first 10 minute interval. The values represented the succinoxidase activity. The side arms containing the ascorbate were then tipped and the oxygen uptake between the 20 to 30 minute interval represented the cytochrome oxidase activity. Correction for autoxidation of ascorbate was made as suggested by Schneider and Potter (8).

In the study of the succinic dehydrogenase by the methylene blue technique the following additions were made to the flasks: 0.50 ml. of 0.21 M phosphate buffer, pH 7.4, 0.50 ml. of 0.5 M sodium succinate, redistilled water to make 3.4 ml. final total volume, 0.1 ml. of 0.15 M potassium cyanide (when used), 0.4 ml. of either 0.01 M or 0.05 M methylene blue (side arm), the α-TPh or SDS solution, and 0.5 ml. of 5 per cent rat heart homogenate.

In all experiments with inhibitors, controls were run simultaneously with the same enzyme preparations because variations were noted in the enzymatic activity of different tissue preparations.

EXPERIMENTAL AND DISCUSSION

Inhibition of Succinoxidase System—The behavior of α-TPh was compared with that of surface-active agents, in particular with dodecyl sulfate. Both α-TPh and SDS inhibited the succinoxidase system of rat liver homogenates at similar low concentrations. Trials at different levels showed that concentrations of 1.9 and 2.8 × 10^{-4} M respectively were just sufficient for complete inhibition, while a concentration of 5.0 × 10^{-5} M of either inhibitor produced only 5 to 10 per cent inhibition. In addition, low concentrations of commercial anionic, cationic, and non-ionic detergents and of fatty acid soaps were found to inhibit succinoxidase. This is in harmony with a recent report of Hockenhull (12).

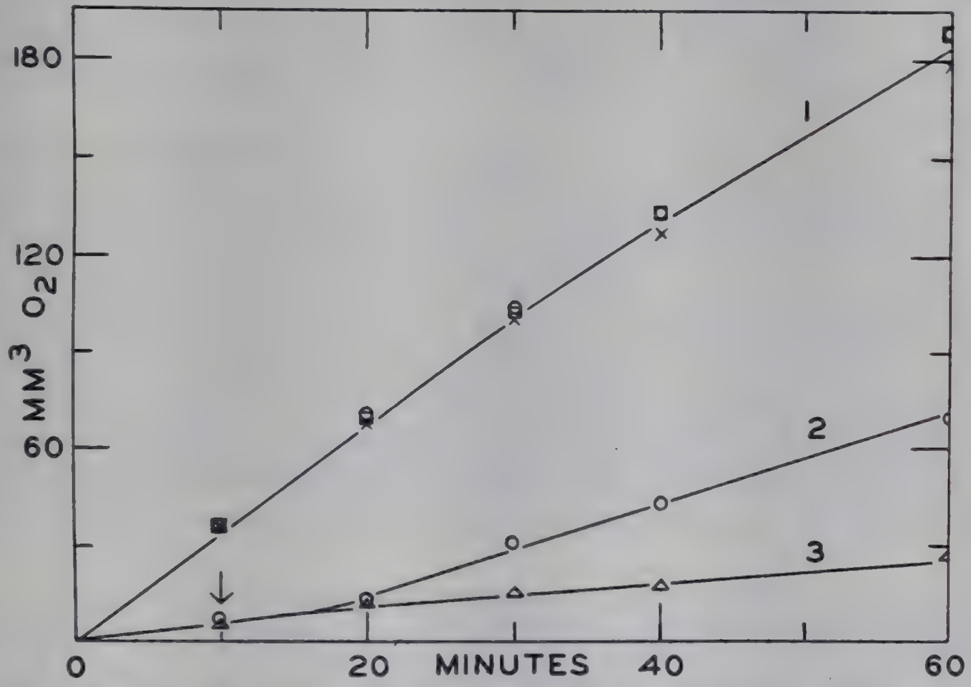

FIG. 1. The effect of heat-inactivated liver homogenate upon the α-TPh inhibition of succinoxidase. Curve 1, ○, succinoxidase assay as described in the text; ×, assay with 0.40 ml. of heated 10 per cent liver homogenate; □, assay in the presence of 1.9 × 10^{-4} M α-TPh to which 0.40 ml. of 10 per cent heated liver homogenate had been added before the active homogenate. Curve 2, as in □ above, but with the heated homogenate added after measuring the oxygen uptake for 10 minutes, as indicated by the arrow. Curve 3, inhibition of succinoxidase by 1.9 × 10^{-4} α-TPh.

A further similarity of the actions of α-TPh and SDS was evident from comparisons of the effect of other proteins on the inhibition produced by these substances. The mixing of α-TPh and of SDS with bovine serum albumin[4] before addition to the flasks decreased their inhibitory effects. Levels of 5 to 7 mg. of albumin per flask decreased the inhibition of α-TPh by about 50 per cent, and that of SDS by about 90 per cent. Serum albumin, in common with a number of proteins, is known to combine readily with anions having large non-polar groups (7, 13). Also, as shown in Fig. 1, the addition of heat-inactivated homogenate to flasks containing sufficient α-TPh otherwise to cause considerable inhibition protected the system completely. Closely analogous results were obtained for SDS inhibition. These observations are best explained by assuming a combination of the α-TPh or SDS with components of the inactivated homogenate, thereby protecting the succinoxidase of the active homogenate added subsequently.

Effect of α-TPh and SDS on Cytochrome c and Cytochrome Oxidase—Since cytochrome *c* and cytochrome oxidase are components of succinoxidase, the inhibition of the system by α-TPh may be a reflection of its effect upon one of these carriers. It has been reported by Jacobi *et al.* (5) that the inhibition may largely be due to an effect on cytochrome *c*, while Houchin (14) found that α-TPh inhibits the reduction of ferricytochrome *c* by ascorbate. Also, in a short discussion of the properties of cytochrome *c*, Keilin and Hartree (15) reported modification of its absorption bands by SDS, and Sevag and Ross (16) found a disappearance of specific cytochrome bands in bacteria upon treatment with zephiran, a cationic detergent.

When either α-TPh or SDS was added to a solution of cytochrome *c* in phosphate buffer, a marked decrease in the characteristic absorption peaks of ferrocytochrome *c* occurred, as shown in Fig. 2. In these experiments, both the cytochrome *c* and the phosphate buffer were in the same concentrations as those used in the manometric experiments with liver homogenate. The effect occurred when the α-TPh or SDS was added either before or after the reduction of the cytochrome. No interference in the reduction was apparent. Concentrations of α-TPh slightly above those required to inhibit the succinoxidase system caused definite decreases in the absorption peaks, but relatively higher concentrations of SDS were needed. Higher concentrations of α-TPh or SDS were effective in reducing the maxima of the absorption bands still more. Although these compounds produced a marked change in the spectrum of ferrocytochrome *c*, a corresponding effect on the oxidized form was not found. Bovine serum albumin, when present as a 2 per cent solution with either the reduced or oxidized cytochrome *c* before the addition of α-TPh or SDS, completely prevented the spectral changes.

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[4] Kindly supplied by Armour and Company, Chicago, Illinois.

In view of the rather striking effect of α-TPh on the absorption spectrum of cytochrome *c*, manometric studies were undertaken to ascertain whether this effect might offer an explanation for the inhibition of succinoxidase. However, as shown in Table I, when a fresh supply of cytochrome *c* was added from the side arm to a system partially inhibited by

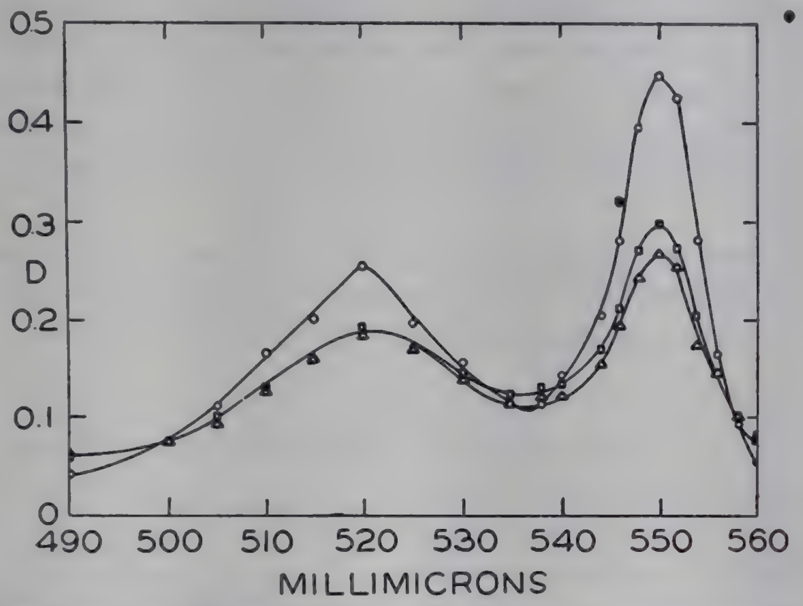

Fig. 2. The effect of α-TPh and SDS upon the absorption spectrum of cytochrome *c*. To test-tubes were added 0.5 ml. of 0.21 M phosphate buffer, pH 7.4, 0.5 ml. of 0.96×10^{-4} M oxidized cytochrome *c*, α-TPh or SDS solutions, and water to make a total volume of 2.8 ml. After incubation at 38° for 15 minutes, 0.2 ml. of a solution of sodium hydrosulfite in phosphate buffer was added to each tube and the absorption spectrum measured with a Beckman spectrophotometer. ○, without α-TPh or SDS, □, in presence of 9.6×10^{-4} M SDS, △, in presence of 2.5×10^{-4} M α-TPh.

Table I

Effect of Additional Cytochrome c, Included after 10 Minutes, upon Succinoxidase Inhibition by α-TPh and SDS

In all cases the initial amount of cytochrome *c* was sufficient to saturate the uninhibited system. With SDS inhibition, although some reactivation was obtained after cytochrome addition, the increase attainable did not approach the value of the uninhibited system.

Succinoxidase source	Initial amount of cytochrome *c*	Amount of cytochrome *c* added after 10 min.	Inhibitor		Oxygen uptake	
			α-TPh	SDS	0–10 min.	20–30 min.
	micromole	*micromole*	$M \times 10^4$	$M \times 10^4$	*c.mm.*	*c.mm.*
Rat liver	0.048	0	0	0	37	36
" "	0.048	0.048	1.35	0	6.8	6.8
" heart	0.090	0	0	0	103	83
" "	0.090	0.090	1.42	0	32	29
" "	0.090	0.090	0	2.84	13	28

α-TPh, no reactivation was obtained. Some reactivation would have been anticipated if cytochrome *c* were made the limiting component. The initial presence of twice the usual amount of cytochrome *c* also did not alter the extent of inhibition. In analogous experiments with SDS, the addition of extra cytochrome *c* only partially reactivated the system. From these experiments it is apparent that the inhibition of the complete succinoxidase system by α-TPh is not due to action on the cytochrome *c* component.

Measurements of the effect of α-TPh on rat heart cytochrome oxidase showed that this enzyme was still active in flasks where the succinoxidase system had been completely inhibited by α-TPh. This was ascertained by the addition of ascorbate from the side arm to such a system, thereby affording a reduction of the cytochrome which was independent of the action of the succinic dehydrogenase. As shown in Table II, at levels of α-TPh or SDS that would completely inhibit the succinoxidase system, the cytochrome oxidase was only partially inhibited. The smaller comparative effect of these inhibitors on the cytochrome oxidase demonstrates that their principal inhibitory action on the complete succinoxidase system is not through an effect on the cytochrome system. It must, therefore, be due either to inhibition of the dehydrogenase or to interference with the interaction of the dehydrogenase and cytochrome systems.

TABLE II

Effect of α-TPh and SDS on Rat Heart Cytochrome Oxidase

For experimental details see the text.

Initial homogenate concentration	Inhibitor concentration	Oxygen uptake	
		Succinoxidase	Cytochrome oxidase
per cent	$M \times 10^4$	*c.mm. O₂*	*c.mm. O₂*
5	0	131	164
5	4.75 α-TPh	0	55
5	4.75 SDS	0	30
1	0	35	75
1	0.36 α-TPh	18	37
1	0.71 "	8	34
1	1.90 "	0	31

Effect of α-TPh and SDS on Succinic Dehydrogenase—The dehydrogenase component or components of succinoxidase may be studied independently of the cytochrome system by use of methylene blue as a hydrogen acceptor. The use of methylene blue in the presence of cyanide to block cytochrome oxidase gave satisfactory oxygen uptakes with heart but not with liver homogenates; hence heart preparations were used in these studies.

The data presented in Figs. 3 and 4 show that methylene blue additions partially restored the oxygen uptake of preparations completely inhibited by α-TPh or SDS. The change from a complete to a partial inhibition was also obtained when potassium cyanide was present in the medium in addition to α-TPh or SDS.

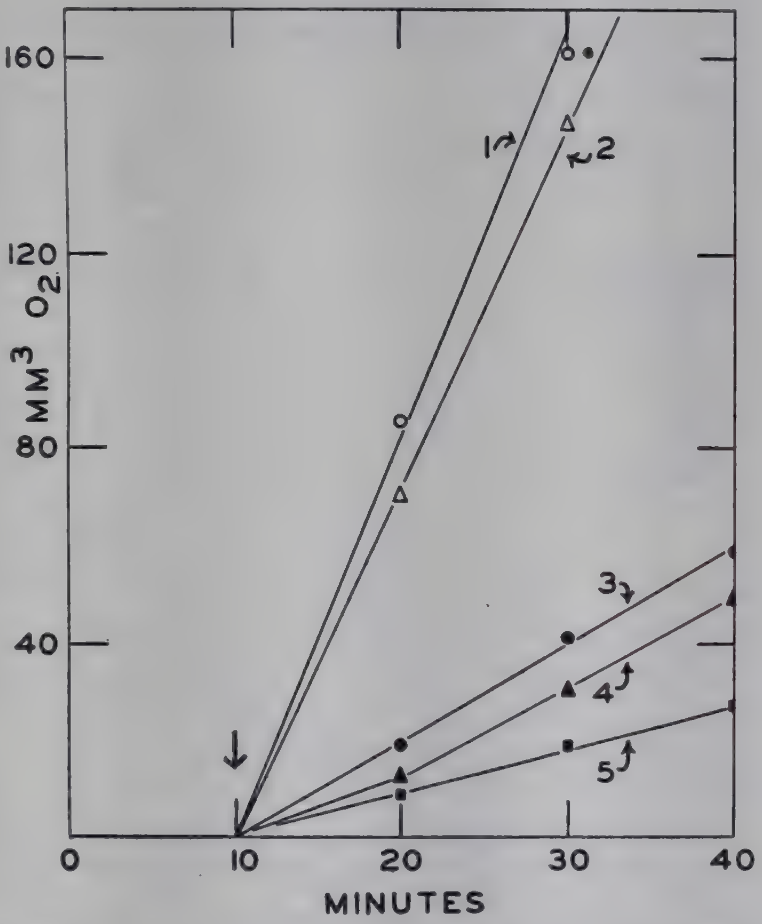

FIG. 3. The effect of methylene blue upon rat heart succinoxidase completely inhibited by α-TPh. Curve 1, methylene blue 6.3 × 10^{-3} M, potassium cyanide 4.7 × 10^{-3} M; Curve 2, methylene blue 1.3 × 10^{-3} M, potassium cyanide 4.7 × 10^{-3} M; Curve 3, methylene blue 6.3 × 10^{-3} M, inhibitor 4.75 × 10^{-4} M; Curve 4, methylene blue 1.3 × 10^{-3} M, inhibitor 4.75 × 10^{-4} M; Curve 5, methylene blue 6.3 × 10^{-3} M, inhibitor 9.50 × 10^{-4} M. For further details see the text.

The possibility existed that the activation of succinic dehydrogenase by methylene blue was due to a combination of the anionic detergents with cationic methylene blue. To ascertain whether this is actually the case, the effect of two levels of methylene blue was determined (Figs. 3 and 4). If the reactivation of the completely inhibited succinoxidase system by methylene blue were due to a removal of α-TPh or SDS from the enzyme, a 5-fold increase in the methylene blue concentration would be expected to decrease the degree of inhibition. However, the degree of inhibition of the dehydrogenase by these inhibitors was the same, within experimental error, for both the low and high methylene blue concentrations. The slightly increased oxygen consumption in all the flasks containing the higher level of methylene blue may be ascribed to an increased saturation of the active centers by this hydrogen carrier.

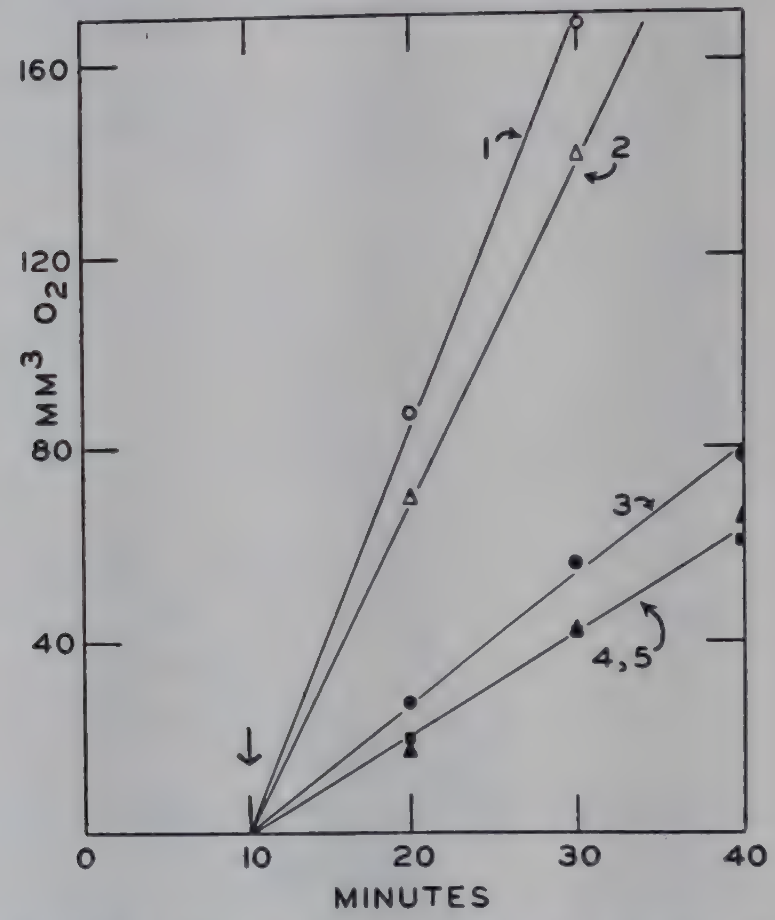

Fig. 4. The effect of methylene blue upon rat heart succinoxidase completely inhibited by SDS. For an explanation of the curves, see Fig. 3.

Interpretation—The partial inhibition of both succinic dehydrogenase and cytochrome oxidase by α-TPh at levels that can completely inactivate the succinoxidase system may be rationalized by the possibility that α-TPh prevents the interaction of the dehydrogenase with cytochrome *c*. Two mechanisms may be suggested.

(1) α-TPh blocks a component linking the dehydrogenase with cytochrome *c*. A number of investigators (17–20) have obtained succinic dehydrogenase preparations which react with methylene blue but not with cytochrome *c*, and have presented evidence for the existence of a factor linking the dehydrogenase system with cytochrome *c*. Ball *et al.* (21)

have explained the inhibition of succinoxidase by the antimalarial naphthoquinone SN-5949 and its reactivation by methylene blue to such a mechanism, while Case and Dickens (22) have reported a similar effect produced by 4,4′-dihydroxystilbene.

(2) α-TPh, when absorbed on the dehydrogenase surface, interferes in the association of the dehydrogenase with cytochrome *c*. It is possible that methylene blue, being a much smaller molecule than cytochrome *c*, can diffuse past these surface barriers to react with the active centers of the dehydrogenase. Keilin and Hartree (11, 23) and Potter (24) have suggested that the configuration of the dehydrogenase essential for combination with cytochrome *c* may be altered by methods used in attempts to demonstrate an intermediate.

Indeed, the former authors (11) have recently critically examined several studies in which intermediates linking the dehydrogenase and cytochrome systems were postulated. They presented evidence indicating that in several cases the activity of a partially inactivated succinoxidase system could be increased by addition of denatured proteins or other materials which present a large surface to the reaction medium. The suggestion was made that the colloidal structure of the preparation affects the mutual accessibility of the enzymes in the system.

Interpretations of the effect of inhibitors of the succinoxidase system in terms of biological function should be made with recognition of the lability of this enzyme to a number of different inhibitors. For example, in addition to the inhibition by α-TPh, the succinoxidase system is known to be inhibited *in vitro* by quinones (21, 25), estrogens (26, 22), narcotics and urethanes (23), lacrimators (27), and other —SH reagents (28), fatty acids (29), and by synthetic surface-active compounds (12).

Previous work has demonstrated that the inhibition of the succinoxidase system by α-TPh might be the result of an accumulation of oxalacetate (4), which acts as a potent competitive inhibitor for succinic dehydrogenase (30). Oxalacetate accumulation is not of major importance in the inhibition by α-TPh and SDS reported herein. This is evident from the partial activity of the dehydrogenase component when the succinoxidase system was completely inhibited and also from the fact that heated liver homogenate, which probably contained both DPN and oxalacetate, partially overcame α-TPh and SDS inhibition. In addition, it was found that when α-TPh, in a level just sufficient to cause 100 per cent inhibition, was added after 10 minutes to a normal succinoxidase system, inhibition was complete in another 10 minutes. Since oxygen uptake was normal during the first interval, it does not seem likely that the subsequent inhibition could have resulted from preservation of any remaining DPN.

The similarity and multiplicity of the effects of α-TPh and of other anions with large non-polar groups do not support the view that the observed effects of α-TPh are a specific function of vitamin E.[5] It is true, however, that they do not preclude the possibility that tocopherol may function as the phosphate ester or a similar water-soluble derivative.

SUMMARY

Inhibition of the rat heart succinoxidase system by α-tocopheryl phosphate is due principally to the prevention of the interaction of the succinic dehydrogenase with cytochrome *c*. Lesser inhibitory effects on succinic dehydrogenase and on cytochrome oxidase are also evident.

The effects of α-tocopheryl phosphate on the components of the succinoxidase system are closely paralleled by sodium dodecyl sulfate. Likewise, both substances decrease the characteristic light absorption of reduced cytochrome *c*.

The results of these studies, together with data already present in the literature, lead to the conclusion that the observed effects of α-tocopheryl phosphate on enzyme systems are due to its properties as an anion with a large non-polar group and are not necessarily related to its action as a vitamin.

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[5] Dr. Stanley Ames, in a private communication, has informed us that he finds that the phosphate esters of γ- and δ-tocopherols have inhibitory effects on the succinoxidase system similar in magnitude to that of α-tocopheryl phosphate, and thus he has concluded that the inhibitory action of α-tocopheryl phosphate is probably not related to the biological function of tocopherol. Dr. Zierler, in a private communication, has also notified us of his conclusion that the *in vitro* action of α-tocopheryl phosphate probably does not reflect the *in vivo* function of tocopherol.

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INTRACELLULAR DISTRIBUTION OF ENZYMES

V. FURTHER STUDIES ON THE DISTRIBUTION OF CYTOCHROME *c* IN RAT LIVER HOMOGENATES

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In a previous paper (1) the distribution of cytochrome *c* in fractions isolated by means of differential centrifugation from suspensions of rat liver in distilled water and in isotonic saline was described. These studies indicated that the cytochrome *c* of the liver cell was concentrated in the large granule (mitochondria) fractions isolated from these liver suspensions. The results were inconclusive, however, because of the poor yields of mitochondria obtained in these fractionations due mainly to incomplete cell rupture as well as to the agglutination of mitochondria in the saline medium.

The discovery that solutions of sucrose prevented agglutination of mitochondria (2) was coupled with a method that permitted almost quantitative breakage of cells (3) in the development of more efficient methods of liver fractionation (2, 4), and hence a reexamination of the distribution of cytochrome *c* within the liver cell was indicated. The results of experiments in which the distribution of cytochrome *c* was determined in fractions isolated from homogenates of rat liver in isotonic and hypertonic sucrose are described in the present report.

EXPERIMENTAL

Materials and Methods

Measurement of Succinoxidase Activity—Succinoxidase activity was determined as described previously (5) by measurement of oxygen uptake in a Warburg apparatus at 38°. Since the rate at which oxygen is absorbed by liver suspensions in the presence of succinate is limited by the amount of cytochrome *c* present, measurements of succinoxidase activity were made in the absence and in the presence of excess added cytochrome *c*.[1] The results are reported as *total* and *specific* succinoxidase activities. The total activities (c.mm. of O_2 absorbed per hour) reported were determined in the presence of excess added cytochrome *c*. The specific activities

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[1] Cytochrome *c* obtained from the Treemond Company and the Sigma Chemical Company was used.

(c.mm. of O_2 absorbed per hour per mg. of total nitrogen) are reported for measurements made in the presence of added cytochrome *c* (Q_s^{+c}) and in the absence of added cytochrome *c* (Q_s^{-c}).

Determination of Cytochrome c—Cytochrome *c* was isolated from the liver homogenates and liver fractions and was determined spectrophotometrically as described by Rosenthal and Drabkin (6).

Preparation of Rat Liver Homogenates—Livers were obtained from adult rats (Sprague-Dawley) that had been fasted overnight to remove liver glycogen. Immediately upon removal the livers were chilled over cracked ice and forced through a masher to remove connective tissue. The liver pulp was weighed and homogenized in 9 volumes of ice-cold isotonic (0.25 M) or hypertonic (0.88 M) sucrose in distilled water. The homogenates were forced through a No. 20 gage hypodermic needle and were then fractionated at 0° in the International model PR-1 and in the Spinco model E centrifuges.

Fractionation of Rat Liver Homogenate in 0.88 M Sucrose—20 ml. of homogenate in 0.88 M sucrose were centrifuged 10 minutes at 3000 R.P.M.[2] (1600 × *g*)[3] to sediment the nuclei, unbroken liver cells, and red blood cells. The sediment was washed once by resuspending in 8.0 ml. of 0.88 M sucrose and recentrifuging at the same speed. The final sediment was resuspended in 0.88 M sucrose and labeled the nuclear fraction, N_w.

The supernatant and washing from the nuclear fraction were centrifuged twice for 10 minutes at 23,000 R.P.M.[4] (29,000 × *g*) to sediment the mitochondria. The sediments were combined and washed twice by resuspension in 8.0 ml. of 0.88 M sucrose and resedimentation for 20 minutes at the same speed. The washed sediment was resuspended in 0.88 M sucrose and labeled the mitochondrial fraction, M_{w2}.

The supernatant and washings from the mitochondrial fraction were combined to form S_1, and 35.0 ml. were centrifuged 60 minutes at 39,460 R.P.M.[5] (130,000 × *g*) to sediment the submicroscopic particles. The submicroscopic particles were washed once by resuspending, diluting to 12.5 ml. with 0.88 M sucrose, and recentrifuging 60 minutes at 50,740 R.P.M.[6] (148,000 × *g*). The washed sediment was resuspended in 0.88 M sucrose and labeled the submicroscopic particle fraction, P_w.

The supernatant and washing from the submicroscopic particles were combined and labeled the supernatant, S_2.

Fractionation of Rat Liver Homogenate in 0.25 M sucrose—20 ml. of homogenate in 0.25 M sucrose were centrifuged 10 minutes at 2000 R.P.M.[2] (700 × *g*) to sediment the nuclei, unbroken liver cells, and red blood cells. The sediment was washed once by resuspending in 8.0 ml. of 0.25 M sucrose and recentrifuging at the same speed. The final sediment was resuspended in 0.25 M sucrose and labeled the nuclear fraction, N_w.

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[2] International horizontal rotor No. 269, 16 × 150 mm. test-tubes.

[3] The *g* values are given for the center of the tubes used.

The supernatant and washing from the nuclear fraction were centrifuged twice for 10 minutes at 9200 R.P.M.[4] (5000 × g) to sediment the mitochondria. The sediments were combined and washed twice by resuspension in 8.0 ml. of 0.25 M sucrose and resedimentation at 20,800 R.P.M.[4] (24,000 × g) for 10 minutes. The washed sediment was resuspended in 0.25 M sucrose and labeled the mitochondrial fraction, M_{w2}.

The supernatant and washings from the mitochondrial fraction were combined to form S_1 and 35.0 ml. were centrifuged 60 minutes at 25,980 R.P.M.[5] (57,000 × g) to sediment the submicroscopic particles. These particles were washed once by resuspending, diluting to 12.5 ml. with 0.25 M sucrose, and recentrifuging 30 minutes at 50,740 R.P.M.[6] (148,000 × g). The washed sediment was resuspended in 0.25 M sucrose and labeled the submicroscopic particle fraction, P_w.

The supernatant and washing from the submicroscopic particles were combined and labeled the supernatant, S_2.

Results

The results of typical fractionations in 0.88 M and 0.25 M sucrose are given in Tables I and II. The values reported are for 20 ml. of homogenate or an equivalent amount of each fraction.

Cytochrome c—The major portion of the cytochrome *c* of the liver homogenate was recovered in the mitochondrial fraction, M_{w2}, regardless of whether the fractionation was made in 0.88 M sucrose or in 0.25 M sucrose. Approximately 50 per cent of the cytochrome *c* of the homogenate was recovered in the mitochondrial fraction in each case. The amounts of cytochrome *c* associated with the nuclear and submicroscopic particle fractions, N_w and P_w, were very low, and the amounts found in these fractions were, in fact, at the lower limits of the method employed for the determination of cytochrome *c*. It was not possible to determine cytochrome *c* in fractions S_1 or S_2 because of the great dilutions of these fractions.

Succinoxidase Activity—The distribution of succinoxidase activity in the rat liver fractions was essentially the same as reported previously (1, 2, 7, 8). The major portion of the succinoxidase activity of the rat liver homogenates was recovered in the mitochondrial fraction regardless of whether the fractionation was made in 0.88 M or in 0.25 M sucrose. It will be noted, however, in contrast to previous reports (1, 2, 7, 8) that only about 80 per cent of the succinoxidase activity of the homogenate was recovered in the fractions in both sucrose solutions. The reason for this low recovery is not known but has been obtained repeatedly in this laboratory. Apparently some activator is present in fraction S_1 because (as will be shown in a subsequent publication) when this fraction is added to the mitochondria the succinoxidase activity is augmented, and when all three fractions (N_w, M_{w2}, and S_1) are combined the succinoxidase activity equals that of the homogenate.

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[4] International multispeed attachment and rotor No. 295, 19 × 63 mm. Lusteroid tubes.

[5] Spinco preparative rotor D.

[6] Spinco preparative rotor A.

TABLE I

Distribution of Nitrogen, Cytochrome c, and Succinoxidase Activity in Fractions Isolated From Homogenates of Rat Liver in 0.88 M Sucrose

Liver fraction	Total nitrogen	Cytochrome *c*		Succinoxidase activity		
		Total	Per mg. N	Total	Q_s^{+c}	Q_s^{-c}
	mg.	γ	γ	*c.mm.* O_2 *per hr.*	*c.mm.* O_2 *per hr. per mg. N*	*c.mm.* O_2 *per hr. per mg. N*
Liver homogenate	72.0	412	5.7	53,400	740	429
N_w	9.5	23	2.4	4,500	470	202
M_{w2}	16.5	212	12.9	29,780	1810	452
S_1	48.8			6,340	130	
P_w	14.1	29	2.1			
S_2	32.2					

TABLE II

Distribution of Nitrogen, Cytochrome c, and Succinoxidase Activity in Fractions Isolated from Homogenates of Rat Liver in 0.25 M Sucrose

Liver fraction	Total nitrogen	Cytochrome *c*		Succinoxidase activity		
		Total	Per mg. N	Total	Q_s^{+c}	Q_s^{-c}
	mg.	γ	γ	*c.mm.* O_2 *per hr.*	*c.mm.* O_2 *per hr. per mg. N*	*c.mm.* O_2 *per hr. per mg. N*
Homogenate	72.9	427	5.9	58,920	820	525
N_w	10.8	43	4.0	7,960	740	356
M_{w2}	16.9	219	13.0	37,080	2200	1010
S_1	47.8			2,420	51	
P_w	17.8	8.5	0.5			
S_2	27.5					

The succinoxidase activity in the absence of added cytochrome *c* (Q_s^{-c}) of the mitochondria isolated from the rat liver homogenate in 0.88 M sucrose was considerably lower than that of the mitochondria isolated from 0.25 M sucrose. Thus the Q_s^{-c} of the mitochondria in 0.88 M sucrose was only 452, whereas in 0.25 M sucrose it was 1010. Since the mitochondria in both 0.88 M and 0.25 M sucrose contained the same amount of cytochrome *c*, it is apparent that the cytochrome *c* associated with the mitochondria in 0.25 M sucrose was much more effective in promoting the oxidation of succinic acid than was the cytochrome *c* associated with the mitochondria in 0.88 M sucrose.

DISCUSSION

The data presented in Tables I and II clearly show that a major portion of the cytochrome *c* of liver homogenates is associated with, and concentrated in, the mitochondria isolated from these homogenates. These data provide additional evidence for the hypothesis that the mitochondria are the centers of respiratory activity in the liver cell inasmuch as previous studies have already demonstrated the association of other respiratory enzyme systems with the mitochondria (1, 2, 4, 7–9). The association of cytochrome *c* with particulate structures such as the mitochondria is of considerable interest because it represents the first instance in which a soluble protein has been demonstrated to be associated with an insoluble portion of the cell. This finding indicates that, although enzymes can be obtained in soluble form from tissues, it does not necessarily follow that the enzymes are present within the cells of that tissue in soluble form.

The cytochrome *c* not associated with the mitochondria is apparently present in the liver cell in soluble form since only about 10 per cent of the total liver cytochrome *c* was recovered in the other particulate fractions of the liver homogenate (N_w and P_w). The possibility must, however, be considered that the entire cytochrome *c* of the intact liver cell is present originally in the mitochondria and partially released during homogenization and fractionation.

SUMMARY

1. The cytochrome *c* content and the succinoxidase activity of fractions isolated by means of differential centrifugation from homogenates of rat liver in 0.88 M sucrose and in 0.25 M sucrose were determined.

2. Approximately 50 per cent of the total cytochrome *c* and 60 per cent of the total succinoxidase activity (determined in the presence of excess added cytochrome *c*) of the rat liver homogenates were recovered in the mitochondrial fraction isolated in either 0.88 M or 0.25 M sucrose. The biological activity of the cytochrome *c* present in the mitochondrial fractions was demonstrated by measurement of the succinoxidase activity of the mitochondria in the absence of added cytochrome *c*.

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METABOLISM OF INORGANIC NITRITE AND NITRATE ESTERS

II. THE ENZYMATIC REDUCTION OF NITROGLYCERIN AND ERYTHRITOL TETRANITRATE BY GLUTATHIONE

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The cardiovascular drugs nitroglycerin and erythritol tetranitrate, while stable at 37° in neutral aqueous solution, decompose in the presence of blood and other tissue preparations to yield inorganic nitrite (1–3). The mechanism of this reductive breakdown has remained obscure. Oberst and Snyder (3) found that heating only partially destroyed the capacity of rabbit liver homogenate to form nitrite from nitroglycerin and postulated the existence of both a heat-stable and a heat-labile factor.

An explanation for these observations has been provided by the results of the present investigation. Reduced glutathione (GSH) was found to undergo a spontaneous reaction with nitroglycerin and erythritol tetranitrate to form inorganic nitrite and oxidized glutathione. GSH could be replaced by cysteine or cysteinylglycine, but not by thioglycollic acid. The reduction of the nitrate esters apparently preceded their hydrolysis, because inorganic nitrate did not react with GSH to form nitrite. It appeared likely that nitroglycerin would be reduced to a nitrite ester which would in turn yield inorganic nitrite and glycerol. However, no glycerol could be determined among the reaction products.

The reduction of nitroglycerin and erythritol tetranitrate by GSH was found to be catalyzed by an enzyme occurring in liver and other tissues. It was purified from hog liver acetone powder by means of fractionation with ammonium sulfate and alcohol, and selective elution from calcium phosphate gel. The preparations were specific for GSH and cysteinylglycine; there was no catalysis of the reduction of nitroglycerin by cysteine. For convenience, the term "nitrite-forming enzyme" will be used.

When homogenates of liver were dialyzed, they no longer formed nitrite upon incubation with nitroglycerin, because GSH was removed. Heated homogenates possessed residual activity, even though the enzyme was inactivated. This was due to the fact that the spontaneous reduction of nitroglycerin by GSH took place at a measurable rate in neutral buffered solutions.

Methods

Measurement of Enzyme Activity—An ethanolic solution of nitroglycerin (Lilly) containing 1.6 mg. of the ester was evaporated in 30 ml. test-tubes by means of a stream of air. Then the tubes were packed in ice and the following additions were made: phosphate buffer (0.067 M, pH 7.4) 1.44 ml., GSH (Schwarz, 0.2 M) 0.08 ml., KCN (0.2 M) 0.08 ml., enzyme, and water to make 2 ml. The tubes were placed in a water bath at 37° and samples removed for nitrite analysis at 5 and 10 minutes. Blank tubes were incubated without enzyme and the rate of the spontaneous reaction was subtracted from the total rate. A *unit* of enzyme activity is defined as that amount catalyzing the formation of 1 μM of nitrite per hour and *specific activity* as units per mg. of protein.

Analytical Methods—Inorganic nitrite was determined in $HgCl_2$ filtrates (4) and inorganic nitrate in phosphotungstic acid filtrates (5). Protein was measured nephelometrically with the Beckman model DU spectrophotometer at 340 mμ (6), by the biuret procedure (7), and with the ultraviolet absorption method of Warburg and Christian (8).

GSH was determined in sulfosalicylic acid filtrates (9). While nitrite interfered with the analysis, this effect could be reduced by adding 1.5 ml. of 0.2 per cent sulfanilamide to 3.0 ml. of incubation mixture just before deproteinization. The recoveries then varied from 67 to 72 per cent, with concentrations covering the range of the data in Table III. Since no way was found to improve this recovery, a correction factor was applied to the analyses.

RESULTS AND DISCUSSION

Non-Enzymatic Reaction between Nitroglycerin and Certain Sulfhydryl Compounds—When nitroglycerin was incubated at 37° in buffered solutions below pH 9, no measurable quantity of nitrite was formed in several hours (Fig. 1, Curve C). However, in the presence of GSH a reaction liberating nitrite took place and its rate increased rapidly as the pH became more alkaline (Fig. 1, Curve A). Almost exactly similar rates were obtained when GSH was replaced by equimolar quantities of cysteine or cysteinylglycine.[1] Thioglycollic acid was inactive. Oxidation of cysteine and GSH during the reaction with nitroglycerin was observed in anaerobic incubations, as indicated by loss of the nitroprusside reaction, decrease in iodine titration, and the appearance of hexagonal crystals of cystine.[2] The oxidized glutathione could be quantitatively reduced with zinc in acid solution.

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[1] Prepared from GSH according to Kendall, Mason, and McKenzie (10).

[2] The crystals had a specific optical rotation $[\alpha]_D^{20}$ of $-214°$ when 26.7 mg. were dissolved in 10 ml. of 1 N HCl. The value for an authentic sample of cystine was $[\alpha]_D^{20} = -206°$. Further identification of the crystals was made by application of the Sullivan reaction, by which a cystine content of 95 per cent was indicated.

When inorganic nitrate was incubated with GSH, no measurable amount of nitrite was formed.

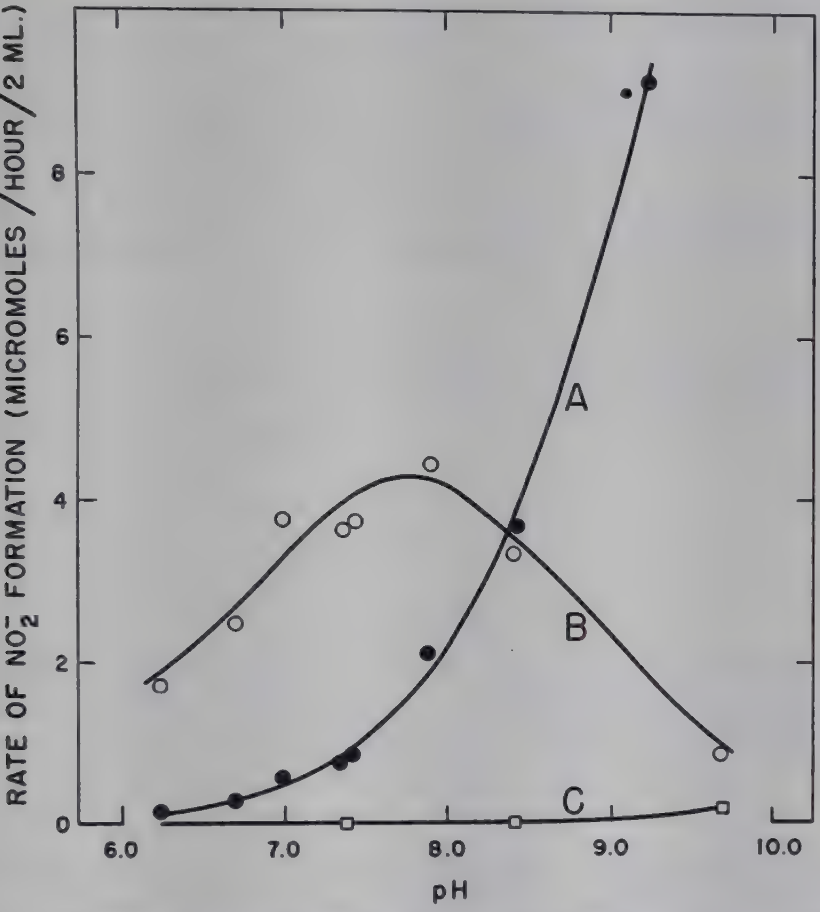

FIG. 1. Formation of nitrite from nitroglycerin and GSH. Curve A, nonenzymatic formation of nitrite from nitroglycerin and GSH. Curve B, rate of the enzyme-catalyzed reaction at different hydrogen ion concentrations. Blank incubation values (without enzyme) were subtracted from the total rate. Curve C, formation of nitrite from nitroglycerin in the absence of both GSH and enzyme. The incubation mixtures contained 78 γ of hog liver enzyme (specific activity 50) and were buffered with 0.05 M phosphate, 0.075 M glycine, or 0.075 M borate.

Enzymatic Reaction between Nitroglycerin and GSH—Nitrite formation from nitroglycerin and glutathione was stimulated when a protein fraction from hog liver was added. Curve B (Fig. 1) shows the rate of the enzyme-catalyzed reaction at different pH values. It was obtained by measuring the total rate and subtracting from it the blank rate (without enzyme). The pH optimum is seen to occur between 7 and 8. With cysteinylglycine the rate of the enzyme-stimulated reaction was about one-fourth that obtained with GSH. There was no catalysis of nitrite formation from nitroglycerin and cysteine.

Nitrite-forming activity was found in homogenates of rabbit lung, stomach, uterus, kidney, and liver, and in several organs of the rat which were examined.

Purification of Nitrite-Forming Enzyme from Hog Liver Acetone Powder

Fresh hog liver, kept for several hours on ice, was homogenized in a Waring blendor with 5 volumes of cold (0°) acetone and filtered on a suction funnel. The procedure was repeated on the filtered cake, after which the material was pressed through a wire screen and dried in a current of air. These operations were performed at room temperature. Subsequent manipulations were at 2°, except as indicated. 56 gm. of acetone powder were suspended in 360 ml. of 0.067 M phosphate buffer, pH 7.4. After a brief interval the mixture was centrifuged for 7 minutes at 13,000 × *g* (crude extract, Table I).

TABLE I

Purification of Nitrite-Forming Enzyme from Hog Liver Acetone Powder

Step	Volume	Total units	Over-all yield	Specific activity
	ml.		*per cent*	*units per mg. protein*
Crude extract	315	13,010		1.1
1st ammonium sulfate fractionation	133	12,900	99	4.3
2nd " " "	85	9,350	72	9.4
Alcohol fractionation	40	4,850	37	37
3rd ammonium sulfate fractionation	35	4,040	31	55
Calcium phosphate, Eluate III	85	1,565	12	80
" " " IV	84	545	4	98

First Ammonium Sulfate Fractionation—The supernatant was adjusted to pH 5.1 with 1 N acetic acid, an inactive precipitate was removed by centrifuging, and solid ammonium sulfate was added to give 0.5 saturation. This precipitate was rejected, and a second precipitate obtained by increasing the ammonium sulfate concentration to 0.9 saturation was collected, dissolved in 0.067 M phosphate buffer, pH 7.4, and dialyzed 3 hours against running demineralized water.

Second Ammonium Sulfate Fractionation—The pH was again adjusted to 5.1, and the precipitate obtained between 0.6 and 0.8 saturation with ammonium sulfate was collected, dissolved, and dialyzed.

Alcohol Fractionation—The dialyzed ammonium sulfate fraction was diluted with distilled water until the protein concentration was 10 mg.

per ml. Then the pH was adjusted to 5.05 and fractions were collected between the following limits of alcohol concentration: 0 to 0.39, 0.39 to 0.54, 0.54 to 0.65. The solution was kept at −8° as soon as danger of freezing was past. The last precipitate contained the bulk of the enzyme activity, and it was dissolved in 0.067 M phosphate, pH 7.4.

Third Ammonium Sulfate Fractionation—The solution in phosphate buffer was treated directly with solid ammonium sulfate to give 0.6 saturation. The precipitate was rejected and the fraction obtained by increasing the concentration to 0.8 saturation was dissolved in phosphate buffer and dialyzed 4 hours against demineralized water.

Adsorption on Calcium Phosphate Gel—The solution was adjusted to pH 6.3 with dilute acetic acid and diluted to give a protein concentration of 0.5 mg. per ml. To every 10 ml. were added 5 ml. of aged calcium phosphate gel (11) (8.2 mg. dry weight per ml.), after which the gel was eluted successively with 10 ml. portions of M/150, M/90, M/45, and M/15 phosphate buffer, pH 7.4. The last two eluates (Eluates III and IV, Table I) showed the best purification.

All fractions were stable for at least several days in the frozen state. The final preparation was stable for 3 weeks at −15°.

Properties of Nitrite-Forming Enzyme

The rate of nitrite formation was proportional to enzyme concentration. Thus, with an alcohol fraction that had 37 units per mg. the following activities in units per ml. of enzyme solution were obtained with different amounts of material: 0.01 ml., 127; 0.03 ml., 136; 0.04 ml., 135; 0.05 ml., 123; 0.06 ml., 123.

It was felt important to rule out possible catalysis by copper salts. Table II shows that cupric sulfate was actually inhibitory and the enzyme-catalyzed reaction was more sensitive than the spontaneous reaction. The enzyme was inactivated by heating to 60°, but was unaffected by 0.1 M sodium fluoride.

The rate of reaction was the same in air and in helium (oxygen-free). A 35 per cent stimulation with 0.008 M cyanide led to its routine use in the enzyme assay. Dialysis for 24 hours against running demineralized water did not alter enzyme activity, nor was the rate of nitrite formation changed by using glycylglycine as a buffer with the omission of phosphate and potassium ions.

Optimal substrate concentrations for the enzyme-catalyzed reaction were 5×10^{-3} M GSH and 3×10^{-3} M nitroglycerin. With excess nitroglycerin the rate was proportional to the concentration of GSH between the limits of 2.5×10^{-4} M and 1.6×10^{-5} M. These observations may provide the basis for a simple and sensitive assay procedure for GSH.

TABLE II

Effect of Cupric Sulfate, Sodium Fluoride, and Incubation at High Temperatures on Reduction of Nitroglycerin by GSH

The incubations in the presence of cupric sulfate were anaerobic, while all other experiments were in an atmosphere of air. Cyanide was omitted from these tests. The incubation mixture contained 78 γ of hog liver enzyme (specific activity 50).

Experimental conditions	Inhibition of enzyme-catalyzed reaction	Inhibition of spontaneous reaction
	per cent	*per cent*
1×10^{-3} M cupric sulfate	88	83
1×10^{-4} " " "	53	4
1×10^{-5} " " "	40	0
1×10^{-1} " sodium fluoride	0	0
Incubation for 5 min. at 70°	100	0
" " 5 " " 60°	50	0

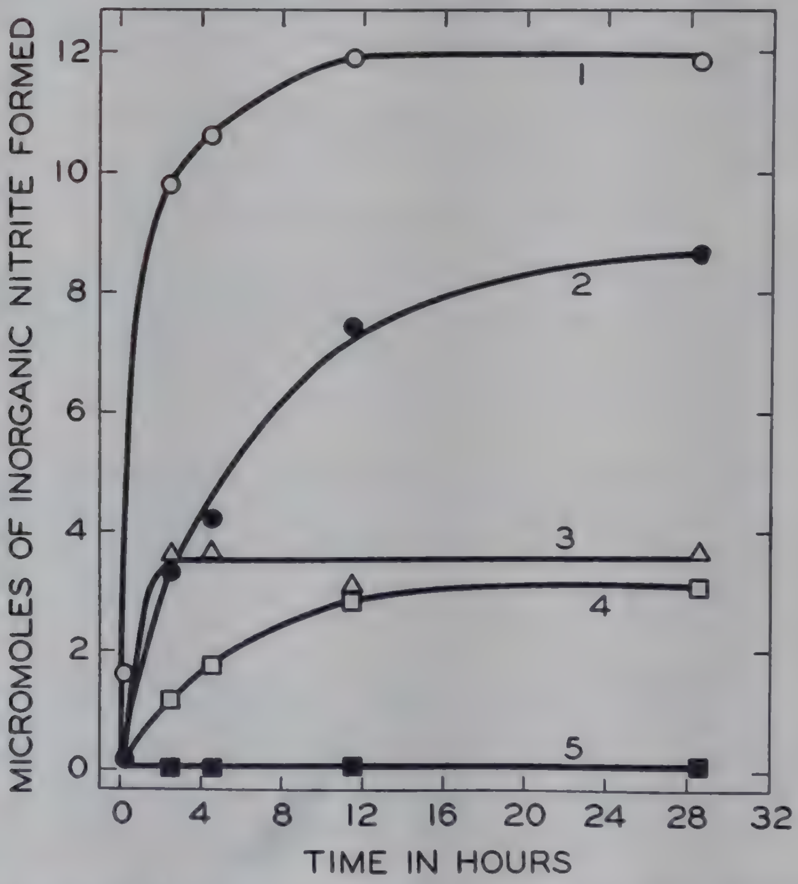

FIG. 2. Formation of inorganic nitrite from nitroglycerin and different amounts of GSH. The incubations were anaerobic, and the usual incubation mixture (see "Methods") was altered by omitting cyanide and increasing the volume to 4 ml. Enzyme addition was 500 γ (specific activity 50). Curve 1, 32 μM of GSH, with enzyme. Curve 2, 32 μM of GSH, no enzyme. Curve 3, 8 μM of GSH, with enzyme. Curve 4, 8 μM of GSH, no enzyme. Curve 5, no GSH.

The purified fractions were tested in several known systems involving glutathione, but the results were negative. They had no glyoxalase activity, did not catalyze the reduction of cytochrome *c* by glutathione, and did not stimulate the oxidation of glutathione in air.

Equivalence of Nitrite Formation and GSH Oxidation

Both the rate of nitrite formation from nitroglycerin and the extent of the reaction were limited by the concentration of GSH (Fig. 2). This suggested that GSH was consumed, which was confirmed by chemical analysis. A balance study was made of GSH disappearance compared with nitrite formation, and the results are shown in Table III. As predicted by theory, 2 µM of GSH were oxidized for every micromole of nitrite formed.

TABLE III

Balance between Nitrite Formation and GSH Oxidation upon Incubation of Nitroglycerin and GSH

The incubations were in Warburg vessels, at 37°, in helium. Total fluid volume, 3 ml.; phosphate buffer, pH 7.4, 0.05 M; nitroglycerin, 3.5×10^{-3} M; GSH, 8×10^{-3} M; 430 γ of hog liver fraction (specific activity 50) added as indicated below. GSH tipped in from a side arm at end of gassing period; duplicate flasks incubated, and results averaged.

Experiment No.	Time of incubation	pH	Enzyme	Glutathione oxidized	Nitrite formed
	hrs.			µM	µM
1	1½	7.0	+	12.4	5.0
	1½	7.0	−	2.0	0.8
2	1½	7.4	+	11.3	5.0
3	1⅓	7.4	+	14.1	7.0
4	16	7.4	+	20.2	8.2
	16	7.4	−	12.8	6.3
5	22½	7.4	+	16.3	7.6
	22½	7.4	−	12.0	5.5

Dependence of Rate of Nitrite Formation on GSH Content of Tissues

Crude liver preparations formed inorganic nitrite upon incubation with nitroglycerin, but this activity was lost after dialysis. The liver enzyme was not destroyed, because additions of GSH restored the nitrite-forming capacity of dialyzed preparations. The reaction rate varied with the amount of GSH added up to the level of substrate saturation. It then became of interest to compare the chemically determined GSH content of crude liver extract with the amount of GSH required to restore the nitrite-forming capacity completely after dialysis.

The experiment was carried out as follows: Fresh rat liver and hog liver acetone powder were extracted with 0.067 M phosphate buffer, pH 7.4. The extracts were tested for rate of nitrite formation in the presence of nitroglycerin and samples were analyzed for GSH. After being dialyzed for 24 hours against demineralized water, the extracts were nearly completely inactive. Varying amounts of GSH were added, and the amount required to restore the rate of nitrite formation to its original level was determined. This amount agreed fairly well with the GSH content of the extracts before dialysis (Table IV).

TABLE IV

Correlation between Amount of Nitrite Formed by Crude Hog Liver and Rat Liver Extracts and Their Content of GSH

A 25 per cent homogenate of rat liver and a 7 per cent suspension of hog liver acetone powder, both in 0.067 M phosphate buffer, pH 7.4, were centrifuged. The supernatant was tested for activity in forming nitrite from nitroglycerin. After dialysis for 24 hours against demineralized water, the activity was nearly completely lost, and it was restored by additions of GSH. The amount of GSH required to obtain full initial activity is compared with the GSH content of the undialyzed extracts.

Experiment No.	Extract	GSH content of extract	Additions of GSH	Nitrite-forming activity	Amount of GSH required to restore activity of dialyzed extracts to original level*
		μM per ml.	*μM*	*units per ml. extract*	*μM per ml.*
1	Hog liver	1.5		4.4	1.5
	Dialyzed hog liver	Trace		0.3	
	" " "		0.75	3.2	
	" " "		1.49	4.1	
	" " "		2.24	5.3	
	" " "		2.98	6.1	
2	Rat liver	2.8			3.1
3	Hog "	1.3			0.9
4	Rat "	2.3			2.4

* Obtained by plotting the nitrite-forming activity of the dialyzed extract against the amount of GSH added to it. From this curve one can read the amount of GSH needed to give an activity equal to that of the original extract. The complete data are shown only for Experiment 1.

Other Reaction Products

The reduction of nitroglycerin by GSH must have preceded hydrolysis, since inorganic nitrate did not undergo conversion to nitrite. The possibility was considered that glyceryl trinitrite was the primary reaction product. This breaks down spontaneously into inorganic nitrite and glycerol (12). Glycerol and inorganic nitrite were reported formed when nitroglycerin was reduced by aluminum in strong acid (13) and by thiophenol in alcoholic KOH (14).

Samples of the incubation mixture were analyzed for glycerol by means of periodate oxidation (15). None was found. In separate experiments glycerol was added in varying amounts. After an incubation period of 16 hours, samples were analyzed for glycerol, and recovery was complete. Apparently this compound was not formed in the reaction between nitroglycerin and GSH or cysteine. Qualitative tests were made for volatile aldehydes which have been reported as products of nitroglycerin hydrolysis in alcoholic KOH (16). Only traces were found.

TABLE V

Formation of Nitrite from Erythritol Tetranitrate

The incubation mixture contained 80 γ of purified hog liver enzyme (specific activity 50). Other additions as described under "Methods," except that, for experiments at pH 9.4, 0.08 M borate buffer was used. The concentration of erythritol tetranitrate (Merck) was 0.2×10^{-3} M.

GSH	Enzyme	pH	Nitrite formed
			μM per hr.
+	+	7.4	3.0
+	−	7.4	0.4
−	−	7.4	0
+	−	9.4	4.6
−	−	9.4	0.4

Inorganic nitrate was formed in a concentration one-fifth that of nitrite in the presence of purified enzyme. This resulted from hydrolysis of the ester or secondary oxidation of inorganic nitrite. During the aerobic incubation of liver homogenate with nitroglycerin much of the inorganic nitrite formed would be oxidized by an enzymatic mechanism described previously (17).

Reaction with Erythritol Tetranitrate

Inorganic nitrite was liberated from erythritol tetranitrate in the presence of GSH, and the reaction was catalyzed by the purified hog liver enzyme preparation (Table V). The non-enzymatic reduction of erythritol tetranitrate by glutathione was 10 times as fast at pH 9.4 as at pH 7.4, just as with nitroglycerin. In the absence of glutathione no measurable nitrite was formed when the ester was incubated at pH 7.4 and the rate was very slow at pH 9.4.

SUMMARY

1. Nitroglycerin and reduced glutathione (GSH) were found to undergo a reaction forming nitrite and oxidized glutathione. Reduction of nitroglycerin preceded hydrolysis, because inorganic nitrate did not react with GSH. The rate of nitrite formation was appreciable in neutral aqueous solution but increased rapidly as the pH became more alkaline. GSH could be replaced by cysteine or cysteinylglycine.

2. An enzyme has been purified from hog liver acetone powder which catalyzes the reaction between nitroglycerin and GSH. It had a pH optimum between 7 and 8 and was sensitive to traces of cupric salts. The enzyme did not stimulate the reaction between cysteine and nitroglycerin.

3. When crude tissue extracts were incubated with nitroglycerin, nitrite was formed. The rate and extent of the reaction could be correlated with the GSH content of these extracts.

4. Erythritol tetranitrate formed nitrite in the presence of GSH, and this reaction also was catalyzed by the protein fraction purified from hog liver acetone powder.

Miss Virginia T. Porterfield assisted in carrying out some of these experiments.

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NEW SYNTHESES OF α-AMINO-ε-GUANIDINO-*n*-CAPROIC ACID (HOMOARGININE) AND ITS POSSIBLE CONVERSION IN VIVO INTO LYSINE*

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α-Amino-ε-guanidino-*n*-caproic acid has a certain interest as a homologue of the amino acid arginine. In view of the metabolic rôles already indicated for the homologues of serine (1), cysteine (2), and glutamic acid (3), it is not inconceivable that "homoarginine" may be found to occur naturally.[1] Some interest in the compound derives also from the fact that synthetic homologues of certain biologically active compounds of diverse types have proved to have physiological activities. Since homoarginine is a derivative of lysine, studies of the former might provide some insight into the metabolism of the latter compound.

Homoarginine has been synthesized by Steib (5) and by Greenstein (6) but studies of its metabolism have been confined to *in vitro* tests of the action of arginase. The data obtained are conflicting; Steib (5) and Greenstein (6) observed no detectable cleavage of the compound, while Felix and Schneider (7) reported a 30 per cent yield of urea in prolonged treatment with the enzyme. Since the probable products of the action of arginase on homoarginine are urea and lysine, it occurred to us that cleavage of the compound *in vivo* to a significant extent might be checked by testing its ability to support growth in rats on a lysine-deficient diet.

In the course of this work, more convenient syntheses of DL-homoarginine have been devised, and the synthesis of the L isomer has been accomplished.

EXPERIMENTAL

Synthesis of DL-*Homoarginine* (*Procedure A*)—This method is based on the procedure of Greenstein (6), with modifications and involves the fol-

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[1] Since this paper was submitted, Dent (4) has suggested that a compound, "fast arginine," detected in certain natural materials by the method of paper chromatography may possess the structure of homoarginine. Our own experiments have indicated that the behavior of homoarginine on paper chromatograms is that of a "fast arginine." It has not yet been possible to make any direct comparison of the materials.

lowing steps: DL-lysine → ε-carboallyloxy-DL-lysine → α-benzoyl-ε-carboallyloxy-DL-lysine → α-benzoyl-DL-lysine → α-benzoyl-DL-homoarginine → DL-homoarginine.

*ε-Carboallyloxy-*DL*-lysine*—This compound was prepared according to the procedure of Stevens and Watanabe.[2]

*α-Benzoyl-ε-carboallyloxy-*DL*-lysine*—By the benzoylation procedure of Carter and Stevens (8), 20 gm. of ε-carboallyloxy-DL-lysine yielded 20 to 21 gm. (69 to 72 per cent) of recrystallized material (from benzene); m.p. 88–91° (uncorrected); N 8.39, 8.27; calculated for $C_{17}H_{22}O_5N_2$, N 8.37 per cent.

*α-Benzoyl-*DL*-lysine*—20 gm. of α-benzoyl-ε-carboallyloxy-DL-lysine, 200 ml. of glacial acetic acid, 8 gm. of palladium-charcoal catalyst (9), and 4 ml. of concentrated hydrochloric acid were placed in a Parr hydrogenation apparatus and shaken under 20 to 25 pounds of hydrogen for 6 hours. The catalyst was removed, fresh catalyst was added, and the hydrogenation repeated. The final mixture was filtered, the filtrate evaporated to a syrup, the syrup dissolved in water, the solution extracted with ether, and the aqueous phase neutralized with ammonium hydroxide. The yield of product, recrystallized from hot water, was 10 to 10.5 gm. (70 to 73 per cent); m.p. 206° (uncorrected). Greenstein (6) reports the melting point at 211°.

*α-Benzoyl-*DL*-homoarginine*—10 gm. of α-benzoyl-DL-lysine were dissolved in 40 ml. of 2 N sodium hydroxide. *S*-Methylisothiourea sulfate (5.5 gm.) was dissolved in 55 ml. of warm water. The two solutions were combined (under the hood) and warmed in a water bath at 65–70° for 15 minutes. Crystals frequently separated during this time. The reaction mixture was allowed to cool for a few minutes, a layer of ether was added, and the flask was stoppered and allowed to remain at room temperature overnight.

The aqueous layer was cooled and the precipitate collected and recrystallized from hot water, yielding 5.8 gm. (50 per cent) of pure material melting at 265° (uncorrected); N 18.82, 18.97; calculated for $C_{14}H_{20}O_3N_4$, N 19.16 per cent. Greenstein (6) reports the melting point at 273°.

DL*-Homoarginine Monohydrochloride*—10 gm. of α-benzoyl-DL-homoarginine were hydrolyzed under reflux for 3 hours with 100 ml. of 5 N hydrochloric acid. The amino acid was isolated as the monohydrochloride by a procedure similar to that employed for the isolation of arginine from benzylidene arginine (10). The syrupy hydrolysis product dissolved in 40 ml. of 95 per cent ethanol was cooled and treated with 4.5 ml. of freshly distilled aniline. The product which separated on cooling was recrystallized from a small volume of water by addition of 95 per cent ethanol. The yield was 5.1 gm. (66 per cent) of material melting at 224°.

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[2] Stevens, C. M., and Watanabe, R., unpublished.

Analysis—$C_7H_{17}O_2N_2Cl$. Calculated. C 37.41, H 7.63, N 24.93
Found. " 37.74, " 7.74, " 24.90

Synthesis of DL-*Homoarginine* (*Procedure B*)—In this method, the copper complex of lysine is treated directly with *O*-methylisourea and the homoarginine isolated from the reaction mixture.

5 gm. of DL-lysine monohydrochloride were dissolved in 20 ml. of water and converted to the copper complex by boiling with 2.5 gm. of cupric oxide, and then removing the excess reagent by filtration. The solution was cooled to 25° and added to a solution of 3.3 gm. of *O*-methylisourea[3] in 6 ml. of water. Sodium hydroxide solution (4 N) was added to pH 10.4. The resulting solution was kept at room temperature for 12 hours and then for 24 hours in an ice box. The crystalline precipitate was collected and the filtrate treated with *O*-methylisourea as before. A much smaller crop of crystals was collected. The combined crops of crystals (4.9 gm.) were dissolved in 50 ml. of warm water and decomposed with hydrogen sulfide. The copper sulfide was removed by filtration with the aid of infusorial earth. The filtrate was acidified to Congo red by the addition of 5 N hydrochloric acid and concentrated to a syrup *in vacuo*. The homoarginine was recovered as the monohydrochloride and purified as described above. The over-all yield from lysine was 3.0 gm. (48 per cent); m.p. 224° (uncorrected); N 24.72, 24.47 amino N (Van Slyke) 6.6; calculated for $C_7H_{17}O_2N_4Cl$, N 24.93, amino N 6.23 per cent.

L-*Homoarginine Monohydrochloride*—This compound was prepared essentially according to Procedure B for the preparation of the DL compound, except that it was found that one treatment with *O*-methylisourea, with the solution adjusted to pH 10.0 to 10.2, gave a yield of 21 to 24 gm. of homoarginine-copper complex from 20 gm. of L-lysine monohydrochloride. When the syrupy product resulting from decomposition of the copper complex and evaporation of the acidified solution was dissolved in 100 ml. of 95 per cent ethanol and treated with aniline (15 ml.), the crude product separated on prolonged cooling. Addition of an equal volume of ethanol produced a second crop, the total yield being 12 to 16 gm. The material was recrystallized by dissolving it in an equal weight of water and adding 4 volumes of ethanol. An additional crop was obtained by adding more alcohol. The total yield was 10 to 14 gm. (40 to 60 per cent); m.p. 207–

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[3] Cyanamide was kindly supplied by Dr. J. T. Cassaday, American Cyanamid Company, Stamford, Connecticut. It was recrystallized and then converted to *O*-methylisourea by the procedure of McKee (11).

209° (uncorrected); $[\alpha]_D^{23} = +18°$ (1.2 per cent in 1 N hydrochloric acid); N 25.12; calculated for $C_7H_{17}O_2N_4Cl$, N 24.93 per cent.

The preparations of DL-homoarginine used in the feeding experiments were shown to be free of lysine by microbiological assay (12) by using *Streptococcus faecalis* and *Leuconostoc mesenteroides*. Under the conditions of the assay, these organisms were apparently incapable of utilizing homoarginine in a lysine-free medium.

The preparations of L-homoarginine used in the feeding experiments were shown to be free of significant quantities (less than 2 per cent) of lysine by chromatographic examination (13) with paper strips and phenol as the solvent in the presence of ammonia. The L-homoarginine was shown to migrate appreciably faster than L-lysine, the approximate R_F values being 0.87 and 0.74, respectively.

Feeding Experiments—Two series of feeding experiments were conducted. In the first group, the ability of DL-homoarginine to support growth on a lysine-deficient diet was tested. Twenty weanling albino rats (Sprague-Dawley) were maintained in separate cages and fed *ad libitum* a basal diet consisting of sucrose 72.0, zein[4] 19.4, finely ground cellulose (Alphacel, Nutritional Biochemicals Corporation) 2.0, salt mixture (14) 4.0, DL-threonine 0.4, DL-tryptophan 0.4, DL-methionine 0.4, L-histidine monohydrochloride 0.4, and DL-valine 0.4 parts respectively. For the first 6 days, all animals were fed the basal diet plus 0.5 per cent of L-lysine monohydrochloride. At the end of this period, they were divided into five groups, each containing two males and two females. The various groups were fed the basal diet, plus the following supplements: 0.5 per cent L-lysine monohydrochloride, 0.25 per cent L-lysine monohydrochloride, basal diet alone, 0.25 per cent L-lysine monohydrochloride plus 1.0 per cent DL-homoarginine monohydrochloride, and 1.0 per cent DL-homoarginine monohydrochloride. Water-soluble vitamins were administered daily as 1 ml. of a solution containing 10 mg. of thiamine, 10 mg. of riboflavin, 10 mg. of niacin, 12 mg. of pyridoxine, 100 mg. of calcium pantothenate, 5 gm. of choline chloride, and 5 gm. of Wilson's (1:20) liver concentrate powder per 500 ml. of solution. 1 drop of halibut liver oil, U. S. P. (Abbott) was given each 4th day. The animals invariably drank the vitamin solutions promptly.

The rats were weighed every 4th day. At that time the food consumed was also measured, and more food was added to the food containers if necessary.

The data obtained in the experiment are recorded in Table I, while the changes in weight of representative animals from each group are shown graphically in Fig. 1. For clarity, and because all groups were remarkably uniform, the data for only one animal from each of the groups receiving lysine supplements are plotted. The rats receiving homoarginine did not respond immediately to the compound, but it will be noted that by the end of 12 days they began to show a definite growth effect. At the end of 28 days, this group of rats was placed on the basal diet with no supplement. At the same time, to the diet of the group which had previously been receiving the basal diet without supplement was added 1 per cent of DL-homoarginine. From the chart, it will be noted that the first group promptly ceased growing. The group now receiving homoarginine showed a definite, but delayed, growth response.

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[4] Obtained through the courtesy of Dr. A. L. Wilson, Corn Products Refining Company, Argo, Illinois.

TABLE I

Results of Growth Experiments with DL-*Homoarginine on Lysine-Deficient Diet*

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		*gm.*	*gm.*	*per cent*
1 ♂	46	1–20	8.5	+2.4	0.5 L-lysine
2 ♂	43	1–20	8.3	+2.1	0.5 “
3 ♀	38	1–20	7.5	+2.1	0.5 “
4 ♀	47	1–20	8.8	+2.4	0.5 “
5 ♂	44	1–20	5.3	+0.7	0.25 “
6 ♂	42	1–20	5.0	+0.5	0.25 “
7 ♀	44	1–20	5.8	+0.7	0.25 “
8 ♀	41	1–20	6.9	+0.9	0.25 “
9 ♂	39	1–28	4.0	−0.2	None
		28–40	3.4	+0.3	1.0 DL-homoarginine
10 ♂	44	1–28	3.5	−0.3	None
		28–40	3.7	+0.1	1.0 DL-homoarginine
11 ♀	50	1–28	4.3	−0.4	None
		28–40	4.2	+0.3	1.0 DL-homoarginine
12 ♀	41	1–28	3.9	−0.2	None
		28–40	3.6	+0.1	1.0 DL-homoarginine
13 ♂	44	1–20	5.1	+0.9	0.25 L-lysine + 1.0 DL-homoarginine
14 ♂	43	1–20	4.8	+0.9	0.25 “ + 1.0 “
15 ♀	43	1–20	5.3	+0.9	0.25 “ + 1.0 “
16 ♀	41	1–20	5.3	+0.7	0.25 “ + 1.0 “
17 ♂	50	1–28	4.0	+0.1	1.0 DL-homoarginine
		28–40	4.7	0.0	None
18 ♂	41	1–28	4.0	+0.1	1.0 DL-homoarginine
		28–40	5.0	−0.2	None
19 ♀	50	1–28	4.2	+0.1	1.0 DL-homoarginine
		28–40	4.5	−0.3	None
20 ♀	42	1–28	4.0	+0.1	1.0 DL-homoarginine
		28–40	4.4	0.0	None

In the second feeding experiment an attempt was made to determine whether the growth response to homoarginine was the result of bacterial action in the intestine by a comparison of the response of different groups of animals receiving the compound by mouth and by subcutaneous injection. Also, higher levels of homoarginine were tested to determine whether the growth response could be increased. In these experiments, L-homoarginine was used.

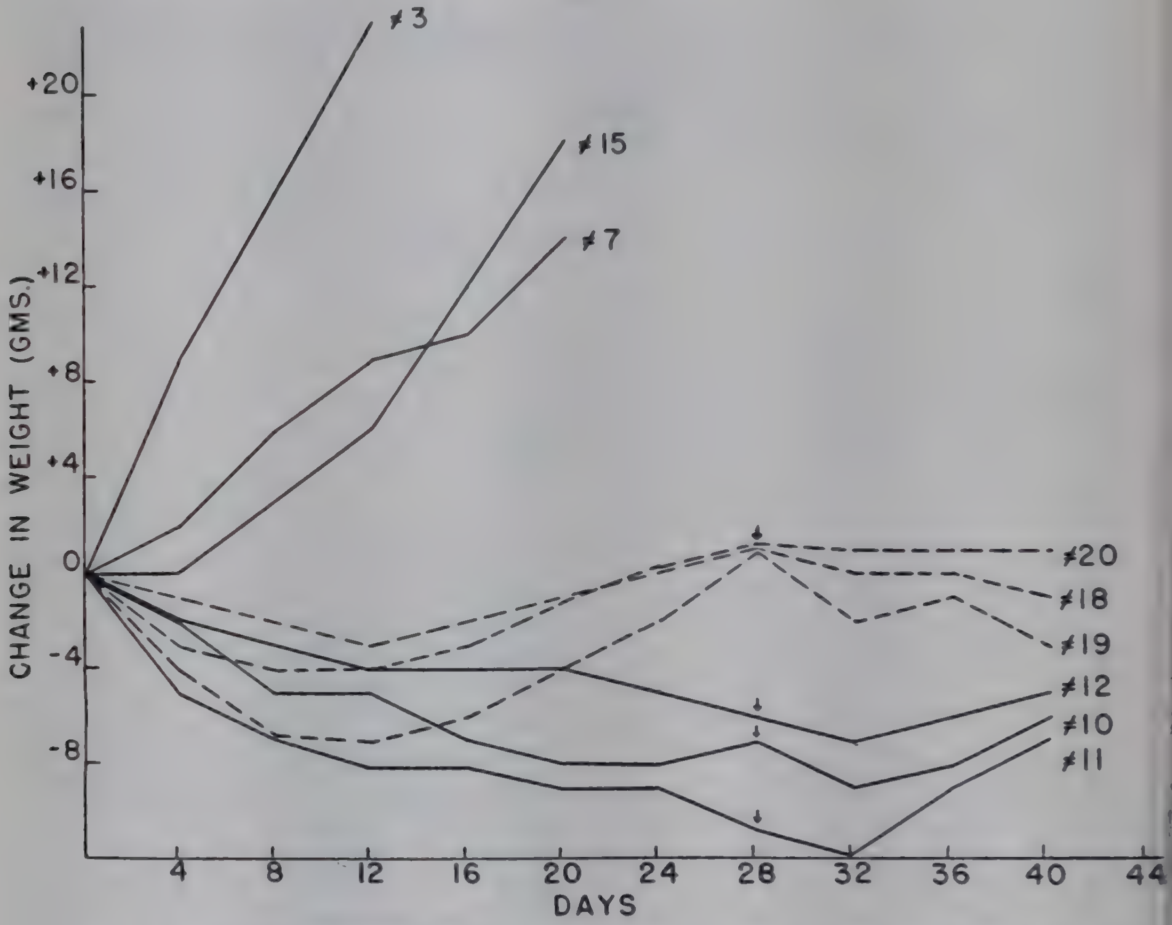

FIG. 1. Changes in weight of rats on a lysine-deficient diet. The small vertical arrows indicate a change of diet. Rats 18, 19, and 20 (broken lines) received 1 per cent DL-homoarginine until the 28th day, when they were placed on the basal diet, while Rats 10, 11, and 12 were placed on the basal diet until the 28th day, when it was supplemented with 1 per cent DL-homoarginine. The remaining rats were fed the following diets throughout: Rat 3, 0.5 per cent L-lysine; Rat 7, 0.25 per cent L-lysine; Rat 15, 0.25 per cent L-lysine plus 1 per cent DL-homoarginine.

Groups of four male albino rats, of the Sprague-Dawley strain 40 days old, were fed the basal diet used in the previous experiment with the following supplements: 0.75 per cent L-lysine monohydrochloride, 0.75 per cent L-homoarginine monohydrochloride, and 1.5 per cent L-homoarginine mono-

TABLE II

Comparison of Growth Response from L-Homoarginine When Fed and When Injected

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		*gm.*	*gm.*	
102	100	1–20	7.6	−0.6	None
118	93	1–20	5.8	−0.7	"
115	87	1–20	6.1	−0.6	"
127	71	1–20	5.6	−0.7	"
109	104	1–16	12.3	+3.7	0.75% L-lysine in diet
112	89	1–16	11.3	+3.6	0.75% " " "
126	90	1–16	11.1	+3.4	0.75% " " "
129	58	1–16	7.5	+2.3	0.75% " " "
110	87	1–20	8.4	+1.2	100 mg. L-lysine per day by subcutaneous injection
113	101	1–20	9.8	+1.0	" "
121	67	1–20	7.5	+1.0	" "
122	90	1–20	8.5	+1.3	" "
105	100	1–8	7.2	−0.6	0.75% L-homoarginine in diet
		9–20	9.8	+1.2	0.75% " " "
111	94	1–8	4.1	−1.5	0.75% " " "
		9–20	6.0	+0.5	0.75% " " "
120	87	1–8	5.0	−0.9	0.75% " " "
		9–20	6.3	+0.4	0.75% " " "
119	73	1–8	4.0	−1.2	0.75% " " "
		9–20	4.8	+0.7	0.75% " " "
101	73	1–8	4.6	−1.0	100 mg. L-homoarginine per day by injection
		9–20	5.3	+0.3	" "
108	96	1–8	6.6	−1.0	" "
		9–20	7.4	+0.2	" "
114	94	1–8	5.8	−1.1	" "
		9–20	6.4	+0.2	" "
123	87	1–8	4.9	−1.1	" "
		9–20	6.3	+0.4	" "
104	96	1–8	5.6	−1.5	1.5% L-homoarginine in diet
		9–20	7.8	+1.1	1.5% " " "
106	86	1–8	5.0	−0.5	1.5% " " "
		9–20	6.3	+0.5	1.5% " " "
107	75	1–8	4.5	−0.9	1.5% " " "
		9–20	5.6	+0.4	1.5% " " "
125	95	1–8	6.5	−1.1	1.5% " " "
		9–20	7.7	+0.7	1.5% " " "
103	96	1–8	5.5	−1.3	200 mg. L-homoarginine per day by injection

TABLE II—*Concluded*

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		gm.	gm.	
		9–20	7.0	+0.3	200 mg. L-homoarginine per day by injection—*Continued*
116	77	1–8	4.0	−1.5	" "
		9–20	6.5	+0.3	" "
117	76	1–8	4.1	−0.9	" "
		9–20	4.8	+0.3	" "
124	95	1–8	5.5	−1.4	" "
		9–20	5.4	+0.9	" "

hydrochloride. Similar groups of animals were fed the basal diet alone but received by subcutaneous injection twice daily one of the following supplements: 25 mg. of L-lysine monohydrochloride, 25 mg. of L-homoarginine monohydrochloride, and 50 mg. of L-homoarginine monohydrochloride. In all other respects, the experiment was conducted in the same manner as the previous one.

The data obtained in the second experiment are recorded in Table II. Among the animals receiving dietary supplements of L-homoarginine, it will be noted that there was again a striking lack of response during the first 8 days, and then a consistent gain through the last 12 days of the experiment. Doubling the level of L-homoarginine in the diet did not increase the rate of gain significantly. The animals receiving subcutaneous injections of L-lysine showed a reasonable rate of growth. The injection of L-homoarginine at the same level gave no response for the first 8 days and then a consistent though rather small growth response. Again, doubling the amount of L-homoarginine injected caused no increase in the growth response of the animals.

DISCUSSION

The syntheses of DL-homoarginine reported in the present paper appear to present definite improvements over published methods. In Procedure A, the use of the more readily available allyl chloroformate and S-methylisothiourea is advantageous, and, in our hands, the isolation of homoarginine as the monohydrochloride is superior to isolation as the sulfate or nitrate. In convenience and over-all yield, Procedure B is still better. By this procedure, L-homoarginine is also readily prepared.

The response to homoarginine in the growth experiments with rats on lysine-deficient diets is rather unique and puzzling. The growth response is delayed and quite small, though consistently observed in every one of a total of twenty-four animals. The limited growth is not due to any effect of the D isomer, since L-homoarginine produces a small response also. Apparently it is not the result of inefficient utilization of the material, since higher levels of the compound show practically identical effects. Previous studies of growth response to derivatives of lysine (15) have shown no evidence of such a delayed effect. One reasonable possibility is that an alteration or adaptation in intestinal flora may represent the mechanism of utilization of homoarginine. However, the similar growth response of animals injected subcutaneously with homoarginine renders this explanation quite unlikely.

SUMMARY

Improved syntheses of DL-α-amino-ϵ-guanidino-*n*-caproic acid ("DL-homoarginine") have been devised. L-Homoarginine has been synthesized for the first time.

Under the condition employed, homoarginine shows a small but definite growth response in rats on a lysine-deficient diet. This is true whether the compound is added to the diet or injected subcutaneously. In both cases, the growth response occurs only after an initial delay of several days.

The authors are indebted to Mr. Gail Stapleton for valuable technical assistance.

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THE EFFECT OF BIXIN AND CAROTENE ON THE OXIDATION OF METHYL LINOLEATE

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While the coupled oxidation of carotene and oils has been studied in some detail (1–7), the rôle of other carotenoids in oxidizing oils is imperfectly understood. Indications that bixin is oxidatively destroyed in a manner similar to carotene were given in the study of lipoxidase by Sumner and Smith (8). Solutions of bixin were also observed to be vastly more stable than similar solutions of carotene. An alcoholic solution of bixin can be kept on the reagent shelf for months without significant deterioration. It was of interest, therefore, to investigate the destruction of bixin in contact with an autoxidizing or a photooxidixing unsaturated oil such as methyl linoleate and to compare the results with those of similar studies of a system containing the relatively unstable carotene. It was believed that such a study might give added information on the mechanism of similar coupled oxidations.

EXPERIMENTAL

The methyl linoleate used in this study was obtained from the Hormel Foundation. Bixin was prepared by hot acetone extraction of annatto powder (Fisher Scientific Company) according to the method described by Sumner and Smith (8). The carotene (General Biochemicals, Inc.) was a 10 per cent α- and 90 per cent β-carotene mixture from a freshly broken ampul.

All oxidations were allowed to take place in Warburg flasks. In the spectrophotometric studies, the flasks were removed at the appropriate times, the contents were diuted with ethanol, and spectrophotometric readings were taken on the dilluted material in the Beckman spectrophotometer. All spectrophotometric data were calculated on the basis of $E^{1\%}_{1\,\text{cm.}}$ of the amount of the carotenoid or the linoleate present in each flask at the beginning of the experiment.

Photooxidation experiments were carried out in a standard Warburg apparatus of the American Instrument Company, modified by G. F. Somers and described by Boyle (9). The water bath was fitted with a plate glass bottom. A series of nine alternating "daylight" and "white" 30 watt fluorescent lamps was mounted approximately 2 inches below the bottom of the water bath. This provided an illumination of about 2000 foot-candles on the bottom of the manometric vessels. In order to prevent moisture condensation on the bottom of the bath and to dissipate some of the heat given off by the lamps, an electric fan was placed so that the stream of air would blow across the surface of the lamps.

Results

In the first series of experiments, the changes in absorption spectra in the bixin-linoleate system were studied. The destruction of bixin was followed by its main absorption band at 457 mμ. It was noted that when 10 per cent of the linoleate had been oxidized, only 1 per cent of the original concentration of bixin could be detected. Carotene has been shown by Holman (7) to act in a similar manner. When the logarithm of the bixin concentration is compared with oxygen uptake (Fig. 1), a linear relationship is evident. It appears, therefore, that this coupled oxidation of bixin is a first order reaction with respect to oxygen uptake as compared to a first order reaction with respect to time. These data indicate that the

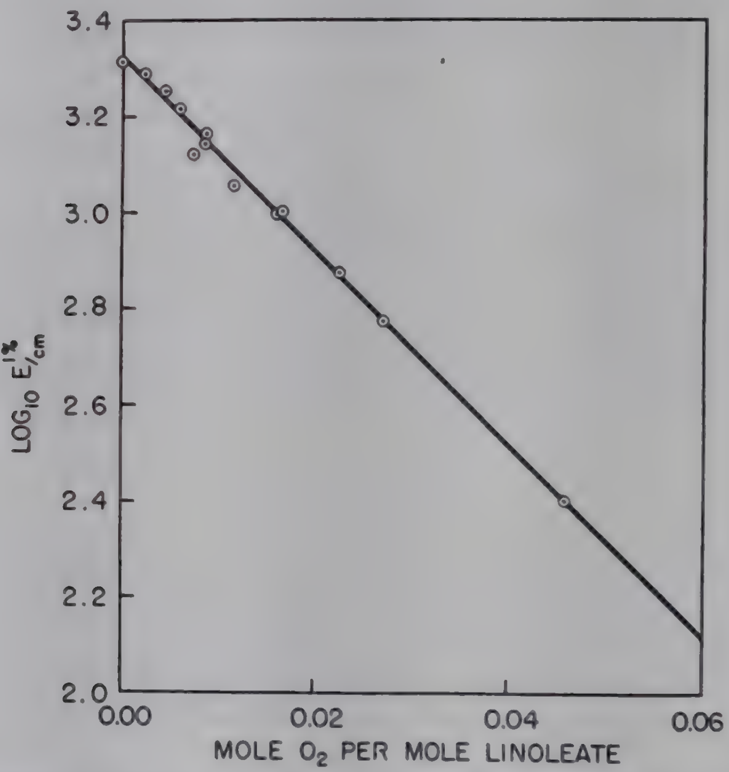

FIG. 1. Logarithm of extinction of bixin (at 457 mμ) compared with oxygen uptake of autoxidizing methyl linoleate. The samples contained 100 mg. of linoleate, 250 γ of bixin, and triacetin to make a total volume of 2 ml. Temperature 25°; gas phase O_2. Total time for experimental run 72 hours. Extrapolation of the curve to 0.10 mole of O_2 per mole of linoleate gives a value of 1 per cent of the original concentration of the bixin.

bixin destruction is a function of oxygen uptake by the linoleate and is probably the result of a reaction with the first oxidation product of the linoleate.

To study the effect of α-tocopherol in the system described above, illumination was used to catalyze the reaction. This allowed a measurable reaction rate without recourse to the addition of catalysts to the flask contents or to raising the temperature of the reaction. The results of the spectrophotometric studies are shown in Fig. 2. It can be seen that the direct relationship of bixin destruction to oxygen uptake does not hold in the early phases of oxidation. It is when the rate of oxygen uptake abruptly increases (see Fig. 4) that the bixin is destroyed. This destruction is as rapid as in the experiments first described. Carotene measured at 448 mμ exhibits a somewhat similar behavior. While the bixin concentration at the end of the induction period was 90 per cent of the original

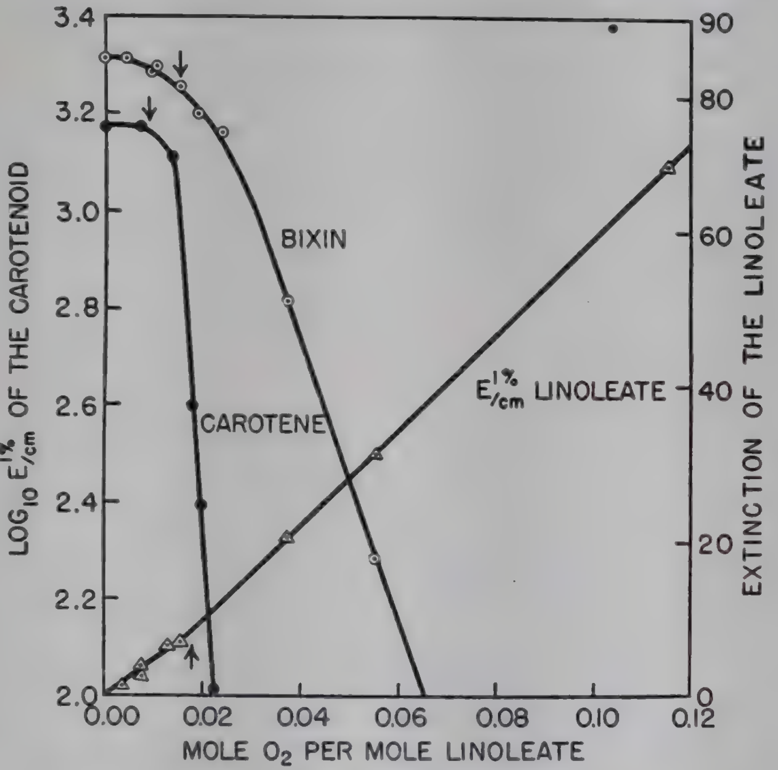

FIG. 2. Logarithm of extinction of bixin (at 457 mμ) and of carotene (at 448 mμ) and absorption of linoleate (at 232.5 mμ) compared with oxygen uptake in photooxidizing tocopherol-methyl linoleate system. The samples contained 100 mg. of linoleate, 250 γ of carotenoid, 125 γ of α-tocopherol, and triacetin to a volume of 2 ml. Temperature 25°; gas phase O_2; illumination approximately 2000 foot-candles. The absorption of linoleate was measured in the presence of bixin. Total time for experimental run, 300 hours for the system containing bixin, 100 hours for the carotene system. The arrows indicate approximate points of the termination of the induction period, that is, the beginning of rapid oxygen uptake (see Fig. 4).

concentration, little or no detectable loss in the amount of carotene chromophores was noted. Diene conjugation in the linoleate was measured at 232.5 mμ. It can be seen to occur even during the induction period. Peroxide formation may be assumed to accompany such conjugation of the unsaturated system (10).

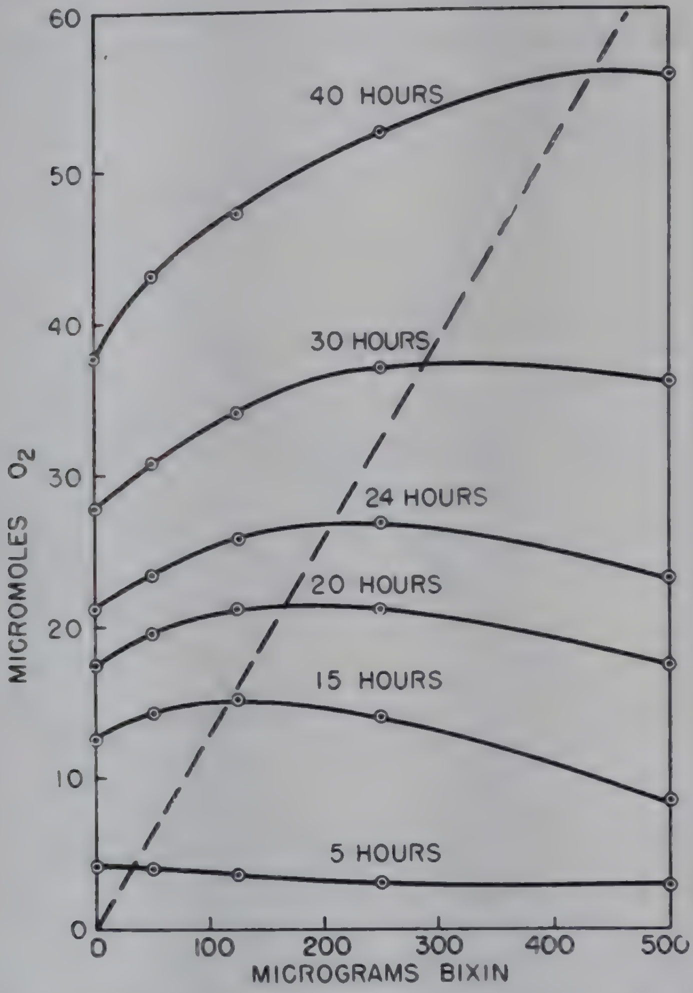

FIG. 3. Oxygen uptake of photooxidizing methyl linoleate in the presence of varying amounts of bixin. The samples contained 100 mg. of linoleate, bixin in the amounts indicated, and triacetin to a total volume of 2 ml. Temperature 25°; gas phase O_2; illumination approximately 2000 foot-candles. Because of the close agreement between duplicates, only average values are plotted. The straight dashed line indicates points of maximal oxygen uptake as the oxidation proceeds.

Preliminary experiments with photooxidation of bixin in dilute methyl linoleate solutions indicated that coupled oxidation of bixin yields a final product which has an absorption maximum at 267 mμ. Attempts to detect intermediates spectrophotometrically during the early phase of oxidation did not give any positive results other than indication of an indefinite transitory increase in absorption between 300 and 315 mμ.

Heftman (1) and Monaghan and Schmitt (5) have reported that small amounts of carotene in an oxidizing system appear to inhibit the oxidation of an unsaturated oil. Increased amounts caused increased oxidation. Ramaswamy and Banerjee (11) have shown that large amounts of annatto powder, which contains bixin in high concentration, exhibit prooxidant activity. In view of this, the effect of varying concentrations of bixin, without tocopherol, on the rate of oxidation of linoleate was studied. In experiments in the dark or dim light, small amounts, 250 γ or less of bixin, added to 100 mg. of linoleate, at times exhibited an antioxidant effect in that the rate of oxidation was decreased. Upon repeated experiments, a great variation in results was obtained and these data do not allow any definite conclusions. Above the 250 γ level, however, there seems to be a definite inclination of increased amounts of bixin to cause greater oxygen uptake by the system. At 48 hours, the oxygen uptakes of 100 mg. samples of methyl linoleate with 0, 500, 750, and 1000 γ of added bixin were 0.9, 3.6, 4.0, and 7.0 μM of oxygen respectively. Here the relative rates of oxidation were established in the early phases and did not later change.

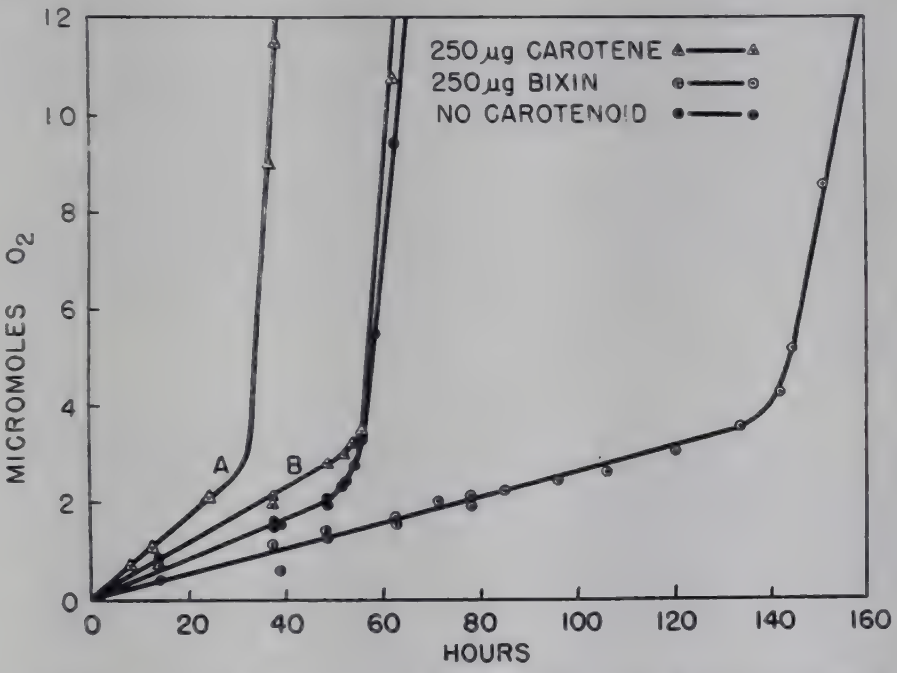

FIG. 4. The effect of carotene and bixin on the rate of photooxidation of methyl linoleate in the presence of α-tocopherol. The samples contained 100 mg. of methyl linoleate, 250 γ of carotenoid as indicated, 100 γ of α-tocopherol, and triacetin to a volume of 2 ml. Temperature 25°; gas phase air; illumination approximately 2000 foot-candles. Curve A for carotene represents the usual oxygen uptake; Curve B represents an example of a slower rate of oxidation during the induction period.

Experiments on the effect of varying amounts of bixin were repeated in the illuminated Warburg apparatus in which a more rapid oxidation was initiated. In the early phases of oxidation under the influence of light, bixin showed a somewhat different action. The larger amounts of bixin actually caused a decrease in the rate of oxidation in the early stages of the reaction. As the reaction proceeded, the increased amounts of bixin then produced a latent acceleration in the rate of oxygen uptake. Representative results are shown graphically in Fig. 3. These effects are highly significant statistically ($P = <0.01$) when 500 γ or more of bixin are present in the system. In the oxidation without lights, the larger amounts of bixin established a prooxygenic activity in the very early phases and this pattern showed no later change.

The rate of oxidation of linoleate with α-tocopherol in the system is also affected by the presence of a carotenoid. Fig. 4 represents an experiment showing the effect of carotene and bixin at the 250 γ level. In all cases, carotene caused a significant increase in the oxidation during the induction period. The length of the induction period was usually shorter than that of the system containing no carotenoid, but in a few cases the times were about the same. Bixin usually produced a slower rate of oxidation but always a greatly extended induction period.

DISCUSSION

The antioxygenic effect of bixin in a photooxidizing system indicates that it may stop the reaction chain as proposed for autoxidizing linoleate (12, 13) by supplying a hydrogen atom without accepting one from linoleate or by an addition of the free radical peroxide to a double bond in the carotenoid. An addition reaction is quite possible, for the well known polymerization tendency in autoxidation of olefinic compounds has been explained by such a mechanism (14).

If the donation of a hydrogen atom occurs, it does not necessarily infer the ensuing destruction of the chromophores. The destruction of both carotene and bixin is delayed in the presence of tocopherol. If addition at the double bond occurs, it would disrupt the conjugated double bond system of the carotenoid. This reaction, therefore, would appear to be inhibited in the presence of tocopherol.

The coupled oxidations of bixin and of carotene in oxidizing oils present certain similarities. Both are largely destroyed in the very early phases of the oxidation. Both carotenoids are protected to some extent by the presence of tocopherol. It is interesting to note the different effects of the relatively labile carotene and the more stable bixin in photooxidation of the tocopherol-linoleate system (Fig. 4). Bixin acted synergistically with the tocopherol to enhance its antioxidant activity. The action of carotene is more difficult to interpret. The effect on the rate of oxidation was a stimulatory one, but the length of the induction period was not consistently shorter than in the solution containing no carotenoid.

Monaghan and Schmitt (5) have obtained evidence to suggest that the prooxygenic activity of carotene is the result of products of decomposition. In the experiments reported here, it may be that some degradation occurred in making up the carotene solution and hence would account for the prooxygenic effect. It is also probable that the carotene was activated by absorbing light energy and that the activated molecule in some manner accelerated the reaction.

The experiments with bixin also indicate that the acceleration in the rate of oxidation is caused by the products of degradation. The relative antioxygenic activity of bixin and carotene appears to depend on the initial rate of oxidation as well as the presence of tocopherol and the concentration of the carotenoid. In the experiments without light, decomposition products appear to exert their influence to a greater degree and the stopping of the chain oxidation by the unaltered carotenoid does not appreciably affect the rate. If the coupled oxidation is catalyzed by light or by lipoxidase (12), the reactions with the unaltered bixin or carotene slow down the oxidation and the effects of the reaction products are delayed.

It appears that, in coupled oxidation, the carotenoid first reacts either by supplying a hydrogen to a free radical peroxide or by adding to the free radical. The secondary reactions appear to produce highly unstable intermediates which may serve in the perpetuation or initiation of chain reactions. The accumulation of such intermediates would therefore not be expected. This is in agreement with the observations in this study and those of Holman (7) in that no such intermediates could positively be detected spectrophotometrically.

The authors' thanks are due to Dr. G. F. Somers of the United States Nutrition Laboratory at Cornell University for the use of the Warburg apparatus modified by him, and to Dr. R. T. Holman of the Agricultural and Mechanical College of Texas for his helpful suggestions in the early phases of this study.

SUMMARY

The relation of spectral changes to oxygen uptake in the coupled oxidation of bixin and methyl linoleate dissolved in triacetin was studied. Bixin is nearly completely decolorized before 10 per cent of the ester is oxidized. The destruction of bixin proceeds as an apparent first order reaction with respect to oxygen uptake as compared to a first order reaction with respect to time. No definite intermediate decomposition products could be detected.

The presence of α-tocopherol inhibits decolorization of both bixin and carotene until the end of the induction period, after which destruction of the carotenoid rapidly takes place.

An antioxygenic effect is exhibited by bixin in the early phases of the light-catalyzed oxidation of linoleate. Also, bixin acts synergistically with α-tocopherol to enhance its antioxidant activity and produces a markedly extended induction period. This activity appears to be caused by the unaltered carotenoid. A latent acceleration of the reaction without tocopherol appears to be the result of the action of decomposition products of bixin.

In photooxidation of the tocopherol-linoleate system, carotene produces an increased rate of oxygen uptake during the induction period.

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THE EFFECT OF ZIRCONIUM AND SODIUM CITRATE ON THE DISTRIBUTION AND EXCRETION OF SIMULTANEOUSLY INJECTED THORIUM AND RADIOSTRONTIUM

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The potential health hazards of radioelements taken into the body are well known (1, 2). The present study is part of a program designed to test the effect of zirconium and other salts on the metabolism of long lived radioelements. It has been shown that the early administration of zirconium salts to experimental animals previously injected with plutonium (Pu^{239}) and radioyttrium (Y^{91}) resulted in a marked increase in the urinary excretion of these radioelements and a considerable reduction of their concentration in bone (3, 4).

Thorium (Th^{230}) and radiostrontium ($Sr^{89, 90}$) are of particular interest because of the marked differences in the nature of their deposition in bone (2). Strontium is closely related metabolically to calcium and the other alkaline earths (2) and is deposited throughout the mineral structure of bone, while many elements, including thorium, plutonium, zirconium, and the rare earths, are deposited primarily in the non-mineralized areas and possibly on the superficial surfaces of the mineralized structure (1, 2). Conditions that affect the metabolism of Ca and Sr do not appear to disturb that of Pu or Y (1, 2). It appeared unlikely, therefore, that zirconium administration would affect the metabolism of Sr.

Until recently there has been little information available on the quantitative distribution and excretion of soluble thorium salts following parenteral administration (2, 6). It has been stated (2) that tracer studies and bone radioautographs showed "no significant differences between the metabolic characteristics of thorium and plutonium." Therefore, it seemed reasonable to expect that the metabolism of injected thorium followed by zirconium administration should be markedly altered, as is the case with plutonium. It is of fundamental interest, therefore, to learn that zirconium has no significant influence on Th metabolism.

Inasmuch as the citrate salt of zirconium was used in our experiments, it was necessary to study the effects of sodium citrate as a control for the action of the citrate ion. Sodium citrate administration is of value in the treatment of experimental uranium poisoning (5) and also of lead poisoning (7), but it exerts little influence on the metabolism of plutonium and yttrium (4).

EXPERIMENTAL

General Procedures—All injections were made by the intraperitoneal route. Twenty female Sprague-Dawley rats, having weights which fell between 197 and 215 gm., were injected with 1.4 ml. of a solution containing the radioisotopes of Th^{230} and $Sr^{89, 90}$. The twenty rats were divided into five groups. To the first group of four rats were administered 4.0 ml. of a zirconium citrate solution containing 12.5 mg. of Zr per ml. $\frac{1}{2}$ hour after injection of the radioelements, while another group of four rats received the same amount of zirconium citrate 3 days later. These groups are referred to as "early zirconium-treated" and "late zirconium-treated," respectively. In similar fashion a third group of four rats received 4.0 ml. of a 5 per cent solution of sodium citrate $\frac{1}{2}$ hour after the injection of the radioelements, and another group of two rats received the same amount of sodium citrate 3 days later. These groups are referred to as "early citrate-treated" and "late citrate-treated," respectively. The last group of six rats, the "untreated controls," received only the radioelement solution. The rats were kept two to a cage. The pooled urine and feces from each cage were collected separately, usually at daily intervals. 8 days after the injection of $Th^{230} + Sr^{89, 90}$ the animals were sacrificed with nembutal, and the liver, pancreas, femur, kidneys, spleen, mesenteric lymph node, and the lungs were removed. The rest of the animal constituted the "carcass" portion.

Radioelement Solution—A small volume of a hydrochloric acid solution of the radioelements was added to a solution of citric acid, the pH was adjusted with NaOH, and the solution diluted with distilled water. After dilution the solution used for injection had the following composition: pH 4, citric acid 1 per cent, Na^+ 0.15 M, $Sr^{89, 90}$ $\sim$15 μc. per ml., and Th^{230} $\sim$0.64 μc. per ml. Each animal received 1.4 ml. of this solution or a total of 1.4×10^6 counts per minute of $Sr^{89, 90}$ and 0.9×10^6 counts per minute of Th^{230}. Inasmuch as the specific activity of Th^{230} is about 43,000 disintegrations per minute per microgram or 21,500 counts per minute per microgram at 50 per cent geometry, each rat received a minimum of $0.9 \times 10^6/21.5 \times 10^3 = 42\ \gamma$ of thorium. Chemical analysis revealed the presence of about 70 γ of Th. This concentration is far below the LD_{50} value (8). A spectrographic analysis of the injection solution showed that, per ml., it contained less than 2 γ of chemical strontium or of cations other than sodium and thorium.

Zirconium and Sodium Citrate—The solution of zirconium citrate, $Na^+[ZrO(C_6H_5O_7)]^-$, was prepared as described previously (4). Each animal receiving zirconium was given 4.0 ml. of a 12.5 mg. per ml. solution or a total of 50 mg. as Zr. The sodium citrate-treated rats each received

4.0 ml. of a 5 per cent solution of sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$. The amounts of Zr or sodium citrate administered were well below the toxic limits (8, 9).

Methods of Analysis—The excreta and tissues were wet ashed by the use of concentrated HNO_3 only. This was accomplished by covering the sample with HNO_3 and evaporating to near dryness on a hot-plate. Fresh acid was then added and evaporated as before. This was repeated from three to eight times, depending on the size of the sample, until no organic matter remained, as evidenced by the light yellow to white color of the ash. Finally, the residue was transferred with HNO_3 to a volumetric flask and diluted to volume (usually to 100 ml. for tissues and to 2000 ml. for carcasses). The carcasses were dissolved by first being covered with concentrated HNO_3, allowed to stand for at least 12 hours, and then treated as described above.

The amount of each radioelement injected into each rat was sufficiently high so that a quantitative measure of its concentration in a sample could be made by direct deposition and subsequent counting of the radiations. Inasmuch as Th^{230} is primarily an α emitter (10) and $Sr^{89, 90}$ a β emitter (10), it was not necessary to effect any separation. For counting Th^{230} an aliquot (usually 0.25 ml.) of an ashed solution was deposited on a platinum disk, slowly evaporated to dryness by means of an infra-red lamp, and flamed. The activity was determined by counting at about 50 per cent geometry in a proportional α-counter unresponsive to β-particles. Samples (usually 5 ml.) for $Sr^{89, 90}$ assay were deposited in a 10 ml. porcelain capsule, evaporated to dryness with an infra-red lamp, and counted by means of a mica window Geiger tube through a 110 mg. per sq. cm. aluminum absorber. The absorber was used to eliminate activity contributed by the accompanying Th^{230} which would otherwise be recorded. The samples were not assayed for $Sr^{89, 90}$ until the Sr^{90} isotope had regained equilibrium with its 65 hour Y^{90} daughter.

The activity in a given sample was always referred to that of samples from the original injection solutions obtained from "dummy" injections into a volumetric flask, which were subsequently mounted and counted under identical conditions. In order to cancel effects due to absorption and other factors, a separate standard for each tissue was prepared by addition of an equivalent amount of an ashed solution of the same tissue from a non-radioactive animal to a sample from the "dummy" injection.

Results

Comparison of Distribution and Excretion of Th^{230} and $Sr^{89, 90}$ in Untreated Rats—The simultaneous administration of the two radioelements permits a direct comparison of their behavior in the same animal. The data tabulated in Table I illustrate the striking differences in the excretion and distribution of Th and Sr after 8 days. Sufficient time having elapsed for the uptake in bone to have reached a maximum (2, 11), it was found that the concentration of injected Th was considerable in all the soft tissues, reaching 30 per cent of the injected dose in liver alone, while that of Sr was negligible, *i.e.* less than 0.02 per cent. On the other hand, the concentration of Sr in the bone was 3 times higher than that of Th.

The total urinary and fecal excretion of Sr was about twice that of Th. Urinary excretion of both radioelements was highest during the first 24 hours after injection. It then dropped sharply during the subsequent days, especially in the case of thorium. By the 8th day the urinary excretion of strontium had reached about 0.4 to 0.5 per cent of the injected dose per day, while that of thorium had diminished to about 0.05 per cent. The day by day urinary excretion of these elements is shown in Fig. 1. The daily fecal excretion of Sr was quite erratic, while that of Th proceeded at a fairly constant rate of about 0.5 to 1.5 per cent a day.

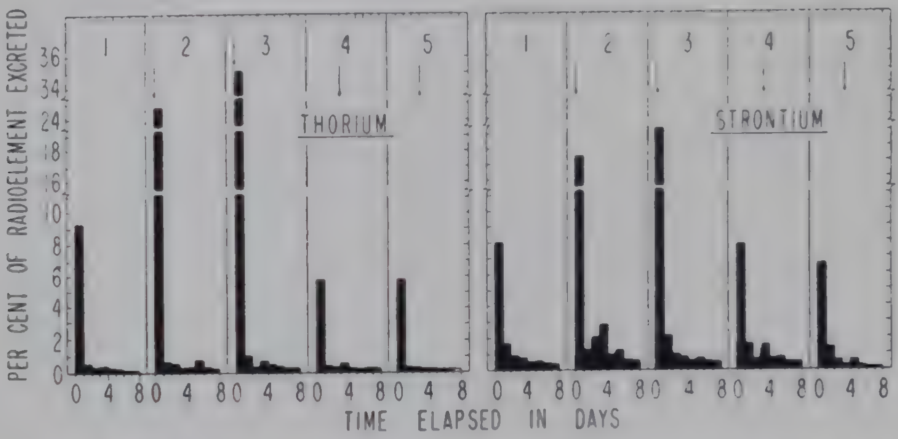

FIG. 1. The effect of zirconium and sodium citrate administration to rats on the urinary excretion of injected Th^{230} and $Sr^{89,90}$, respectively. Each arrow represents a single intraperitoneal injection of zirconium or sodium citrate. The numeral within each panel represents the following treatments: Panel 1 controls, Panel 2 early zirconium citrate, Panel 3 early sodium citrate, Panel 4 late zirconium citrate, Panel 5 late sodium citrate.

Effect of Treatment—The early administration of either zirconium or sodium citrate caused the urinary excretion of Th to increase from about 9 to about 30 per cent of the injected dose (Fig. 1) and that of Sr from 8 to about 18 per cent during the subsequent 24 hour period (Fig. 1). Some sustained increase in Sr excretion following early Zr administration, however, seems to have occurred. Injections of sodium citrate 72 hours after exposure to Th and Sr had no effect on their urinary output, while zirconium citrate appeared to have a slight effect on Sr excretion and none on Th (Fig. 1). It must be kept in mind, however, that a 3-fold increase, for example, in the urinary excretion of a radioelement shortly after exposure usually constitutes an appreciable fraction of the body content but is relatively unimportant when the normal excretion rate has dropped to very low levels.

The excretion of Sr or Th in the feces was unaffected at any time by the administration of zirconium or sodium citrate.

Since the major part of the increased excretion of the thorium which followed either early sodium or zirconium citrate administration came from the liver, it must be concluded that the citrate ion is responsible for the observed effects (Fig. 2 and Table I). Thorium excretion was also accomplished at the expense of the other soft tissues, namely the kidneys, spleen, mesenteric lymph node, and pancreas, following the administration of sodium citrate. The increased strontium excretion seemed to come from the skeleton primarily. It appears likely that the muscle and skin contributed some of the increased Sr excretion inasmuch as these tissues *in toto* contain about 35 per cent of tracer Sr 30 minutes after intraperitoneal injection (12).

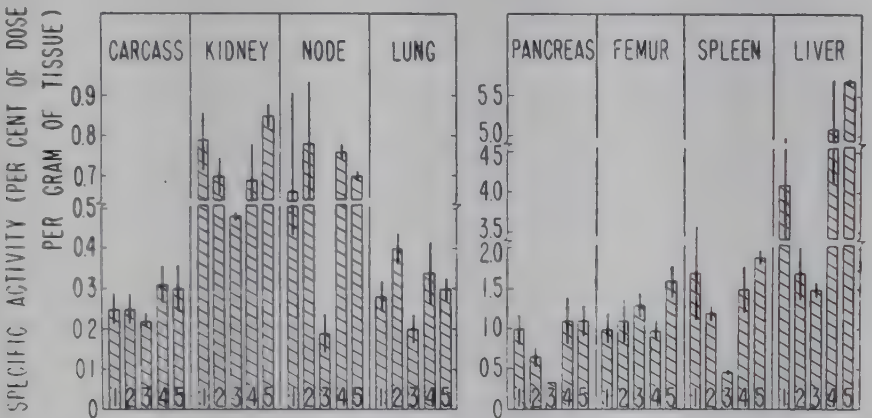

FIG. 2. The effect of zirconium and sodium citrate administration on the concentration of injected Th^{230} in the tissues of rats. The rats were sacrificed 8 days after receiving the Th^{230}. The numerals inside the panels represent the following treatments: Panel 1 controls, Panel 2 early zirconium citrate, Panel 3 early sodium citrate, Panel 4 late zirconium citrate, Panel 5 late sodium citrate. The vertical lines represent the average deviation among the members of each group of rats.

The results described above are in marked contrast to those obtained with Pu under the same conditions (4). Thus, early zirconium citrate administration increased the urinary excretion of Pu from the normal 1 to 50 per cent of the injected dose during the subsequent 24 hours, while sodium citrate caused a 2-fold increase only. In addition the bone content of Pu was found to be reduced about 6-fold as a result of zirconium treatment, while the soft tissue concentrations were essentially unchanged. Furthermore, the administration of zirconium citrate 3 days after the injection of Pu raised the urinary excretion level from 0.1 to 5 per cent of the injected dose.

TABLE I

*Effect of Zirconium and Sodium on Distribution and Excretion of Simultaneously Injected Thorium and Radiostrontium in Rats**

	Per cent injected dose per organ									
Tissues	Untreated controls		Early zirconium, citrate-treated		Early sodium, citrate-treated		Late zirconium, citrate-treated		Late sodium, citrate-treated	
	Th	Sr	Th	Sr	Th	Sr	Th	Sr	Th	Sr
Carcass	47.4 ±6.9	67.0 ±5.4	48.2 ±6.2	53.0 ±4.4	41.0 ±2.8	56.9 ±9.9	51.1 ±9.2	66.0 ±4	49 ±6	70.5 ±3.3
Skeleton†	17.8 ±3.6	52.8 ±6	20 ±2	40.8 ±4.1	22 ±4	44.6 ±10	17.2 ±1.8	46.6 ±2	28 ±1	50 ±0.2
Liver	29.6 ±6.8	0.015 ±0.002	14.4 ±2.3	0.020 ±0.006	9.8 ±2.0	0.010 ±0.001	31.8 ±4.5	0.018 ±0.002	33.8 ±1.8	0.020 ±0.002
Kidneys	1.2 ±0.08	<0.02	1.1 ±0.12	<0.02	0.74 ±0.12	<0.02	1.0 ±0.17	<0.02	1.2 ±0.1	<0.02
Spleen	1.0 ±0.2	<0.01	0.66 ±0.02	<0.02	0.27 ±0.01	<0.01	0.85 ±0.12	<0.02	0.82 ±0.06	<0.02
Mesenteric lymph node	0.30 ±0.06	<0.01	0.41 ±0.10	<0.01	0.12 ±0.08	<0.01	0.29 ±0.02	<0.01	0.26 ±0.20	<0.01
Pancreas	1.0 ±0.2	<0.01	0.61 ±0.10	<0.01	0.33 ±0.08	<0.01	1.1 ±0.3	<0.01	0.93 ±0.20	<0.01
Lung	0.37 ±0.04	<0.02	0.53 ±0.04	<0.02	0.29 ±0.04	<0.02	0.45 ±0.10	<0.02	0.43 ±0.09	<0.02
Urine	10.6 ±4	13.5 ±4	27.4 ±6	27.1 ±8	36.8 ±3	25.1 ±5	6.3 ±0.5	14.2 ±0.5	6 ±3	9.70 ±4
Feces	7.7 ±2	16.7 ±1.5	5.5 ±2	19.0 ±2.1	9.6 ±3	16.2 ±2.2	6.2 ±2	17.5 ±0.6	6 ±3	17.4 ±3

* Values equated to 100 per cent (actual recoveries for Th = 94.5 ± 8.2 per cent, and for Sr = 90 ± 4 per cent). The rats were sacrificed 8 days after the intraperitoneal administration of Io^{230} (Th) and $Sr^{89, 90}$. Each value is an average of results obtained from six rats for the untreated controls, four rats each for the early sodium citrate, early zirconium citrate, and late zirconium citrate-treated animals, and two rats for the late sodium citrate group. Further details are given in the experimental sections of the text.

† The concentrations of radioelement in the skeleton were calculated by multiplying the femur content by the somewhat arbitrary factor of 20.

DISCUSSION

Action of Sodium Citrate—The action of early sodium citrate administration in minimizing the deposition of thorium in the soft tissues with a resulting increase in the urinary excretion is related to the complexing action of the citrate ion accompanied by a concomitant increase in the diffusibility of the Th. Most cations in the body exist as a mixture of diffusible and non-diffusible forms. The non-diffusible fractions result from a combination of the cation with proteins, or because of the formation of particles with colloidal properties as a consequence of hydrolysis or reaction with anions to form salts of low solubility. An increase in the diffusible fraction at the expense of the non-diffusible parts is reflected in a lower deposition in the soft tissues comprising the reticuloendothelial system and an increase in the urinary excretion because the rate of filtration through the glomerulus of the kidney is enhanced. These considerations are supported by the fact that citrates form soluble complex ions with thorium (13) and by ultrafiltration studies in which the passage of Th from blood serum through Visking membranes was markedly increased following the addition of sodium citrate.[1] Similar concepts have been proposed to explain the action of sodium citrate and other complexing axions in the metabolism of uranium (14, 15) and lead (16).

Once an element such as thorium, which precipitates from simple inorganic solution above a pH of 3.5, has been deposited in the body, it is very difficult to increase its subsequent rate of elimination. This is probably related to the well known chemical fact that very large (unphysiological) amounts of the citrate anion are needed to dissolve the hydroxide of an element such as Th once it has formed. This phenomenon explains the relative ineffectiveness of sodium citrate in promoting the excretion of Th when administered several days later.

Of further interest is the fact that the concentration of injected Th in the spleen, mesenteric lymph node, and pancreas was clearly unaffected by early zirconium citrate administration; yet early sodium citrate administration caused a marked reduction. Such a result is probably related to the fact that in zirconium citrate the citrate part of the molecule is not immediately available because it is bound to zirconium. Thus in the time necessary for the zirconium citrate to be absorbed following intraperitoneal injection, the mesenteric lymph node, spleen, and pancreas are not in contact with free citrate. Subsequent metabolic processes free the bound citrate, whereupon it is available to exert a solubilizing effect through complexing action on the Th. Free citrate, however, is lost in the liver partially by conversion to glycogen (17).

The uptake of strontium and the other alkaline earths as well as the phosphate ion is most easily interpreted as being an ion exchange reaction (18–20) with the bone acting as either an anion or cation exchanger containing a variety of functional groups. We can represent the cation exchange with strontium by the relation

$$\mathrm{Ca}A + \mathrm{Sr}^{++} \rightleftharpoons \mathrm{Sr}A + \mathrm{Ca}^{++} \tag{1}$$

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[1] Unpublished experiments.

in which, for simplification, we assume that A is a composite anion of all the functional groups in bone with a valence of -2. The capacity of bone for cation exchange can be expressed in terms of the total milliequivalents of replaceable calcium. The distribution coefficient, K_d, for a given cation adsorbed by bone is given by the expression (21)

$$K_d = \frac{M_s}{M_1} \times \frac{v}{m} \tag{2}$$

in which M_s and M_1 are the fractions of the adsorbed cation in the bone and liquid phases respectively, and v is the volume of solution in contact with m mg. of bone. The ratio $M_s{:}M_1$ is expressed conveniently as (per cent adsorbed)/(100 per cent minus per cent adsorbed). By employing the same reasoning used with resinous cation exchangers (21), it can be shown that, to a first approximation, K_d is directly proportional to the mole fraction of exchangeable Ca^{++} in bone. Therefore K_d, and hence the amount of $Sr^{89,90}$ exchanged, will remain constant as long as the mole fraction of Ca and other exchangeable cations in bone remain unchanged. Since a gm. of bone contains about 1 mM of exchangeable calcium, tracer levels of $Sr^{89,90}$, *i.e.* $\sim 10^{-10}$ M, cannot affect K_d, regardless of the extent to which the tracer Sr is adsorbed. In the *in vitro* experiments of Falkenheim *et al.* (19), 50 mg. of bone ash were equilibrated with 25 ml. volumes of solution-containing Ca^{++}. Their bone sample presumably contained about 0.05 mM of exchangeable calcium. When the contacting solution contained less than 10^{-4} M, *i.e.* <0.0025 mM of tracer alkaline earth, no appreciable change in K_d could be expected. However, a solution 10^{-3} M, equivalent to 0.025 mM, if adsorbed to the same extent as tracer levels, ~ 75 per cent or more as found for Sr and Ca (19, 22), would markedly reduce the mM of Ca in bone, and hence a sharp drop in the per cent adsorption must take place at about 10^{-3} M, in agreement with the experiments on bone (18, 19).

The preceding calculations explain why the injection of 0.01 to 10 μc. of carrier-free $Sr^{89,90}$ in the experiments of Murray and Bloom (23) resulted in no change in the per cent uptake.[2] In some experiments of Copp and Greenberg (24) it was thought that the injection of non-radioactive "carrier" strontium might dilute and wash out radioactive strontium. In view of the large amount of exchangeable calcium in bone it can be calculated from equation (2) that it would require about 60 mg. of Sr in a 200 gm. rat to affect the uptake of Sr appreciably. This was, indeed, found to be the case, but inasmuch as this amount of Sr is lethal or near lethal it is of no practical value.

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[2] We are, of course, disregarding any effects due to the radiation itself.

Elements such as Th, Pu, and Zr, because of their chemical properties, are most probably retained by bone through an irreversible type of nonspecific surface adsorption. Therefore, the uptake and elimination of these elements by bone, in analogy to similar inorganic systems (21), should be unaffected by changes in bone resulting from age, diet, or other ordinary metabolic changes. A fuller discussion of these points together with pertinent experimental data will be given in a subsequent paper.

SUMMARY

1. The distribution and daily excretion of tracer levels of injected thorium and strontium were studied in rats receiving zirconium or sodium citrate intraperitoneally $\frac{1}{2}$ hour after the administration of the radioelements ("early" treated) or 3 days later ("late" treated). One group of rats received no other treatment.

2. In contrast to previous studies on plutonium and yttrium, zirconium citrate had no specific effect on Th or Sr metabolism other than that associated with the citrate part of the molecule.

3. The early administration of zirconium or sodium citrate resulted in about a 3-fold increase in the urinary excretion of Th and a 2-fold increase in the urinary excretion of Sr during the following 24 hours. No effect on the fecal excretion was found. The Th concentration in the liver, mesenteric lymph node, pancreas, and spleen was markedly reduced as a result of early sodium citrate administration. No significant changes in the metabolism of Th or Sr were found in the late treated groups.

4. The deposition of Th per gm. was highest in the soft tissues, particularly in the liver, while Sr accumulated mainly in the skeleton.

5. An interpretation of the results in terms of ion exchange, surface adsorption, and complex ion formation was presented.

We wish to express our appreciation to Rosie Hunter for the mounting and counting of many of the numerous samples required by this work.

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GROUPS INVOLVED IN THE ZIMMERMANN AND KOBER REACTIONS

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Colorimetric methods for the quantitative determination of many compounds have been developed, based on their specific chemical or physicochemical properties. Such tests include the Zimmermann (1) reaction for androgenic assay and the Kober (2) reaction for estrogenic assay. Modifications of each of these have been reported by Callow *et al.* (3), Cohen and Bates (4), Szego and Samuels (5), and Holtorff and Koch (6). Many of these modifications are for the determination of estrogens and androgens obtained from the extract of urine, feces, and body tissues. The value of these variations lies in the fact that certain groups inherent in the perhydro-1, 2-cyclopentanophenanthrene nucleus or its derivatives are involved. The color development in the Zimmermann reaction (Zimmermann (1) and others) depends upon a carbonyl group adjacent to a methylene carbon ($-CH_2-CO-$). For this reason many compounds not related to 17-ketosteroids will give positive results. Callow (3) has implied that the group $-CH_2-CO-CH_2-$ found in cyclopentanone is necessarily the one found in perhydro-1, 2-cyclopentanophenanthrene compounds giving a positive Zimmermann. If this were true, only estrone-16 would give a positive reaction. These data do not confirm this.

Marrian (7) has postulated that 16-ketoestrone is the basis for the red color developed in the Kober reaction, and that only α-diketones could give a positive Kober test. This was before 16-ketoestrone was synthesized and tested. Marlow (8) showed that little or no color is developed when 16-ketoestrone is treated with the Kober or Zimmermann reagent as compared to other estrogens.

EXPERIMENTAL

The compounds used in these experiments are pure crystalline compounds of proved structure. The quantity treated with the Zimmermann reagent has an arbitrary concentration of 72 γ, while that treated with the Kober reagents has 30 γ. Some of these compounds (estrone-16, 16-ketoestrone, etc.) have not been reported as subjected to the Kober and Zimmermann tests, since they have only been synthesized and their structures established very recently. These compounds are critical ones structurally for the determination of groups involved in the color development of the Kober and Zimmermann reagents. The other compounds have been reported but are included for comparative effects.

The methods involved necessitated only minor modifications in those cited in references above. For clarification they are briefly outlined below.

Zimmermann—0.2 ml. of solutions (0.2 ml. of redistilled 95 per cent alcohol as blank) containing 72 γ was treated with 0.2 ml. of 5.5 N KOH and 0.2 ml. of 2 per cent *m*-dinitrobenzene in alcohol. The tubes were stoppered and allowed to stand for 45 minutes. They were then diluted with 10 ml. of 70 per cent alcohol solution.

Kober—1.0 ml. of an alcohol solution (1 ml. of redistilled alcohol as blank) containing 30 γ of the substance to be examined was evaporated to dryness in a hot water bath. 0.6 ml. of a fresh, modified Kober reagent (0.625 gm. of β-naphthol dissolved in 25 ml. of concentrated H_2SO_4) was added. The tubes were heated for 2 minutes (shaking at 45 and 90 second intervals) in a boiling water bath and then cooled in ice water for a few seconds. 0.6 ml. of acidulated water was added with agitation while the tubes were in the ice water. They were then reheated in boiling water for 1½ minutes, being shaken well at 45 seconds. The tubes were transferred to an ice water bath for 10 seconds. Then 1.8 ml. of 65 per cent H_2SO_4 (1:1) were added. The solutions were mixed well and then transmission was determined in the Cenco-Sheard spectrophotelometer.

Spectrophotelometer—The instrument was set to give 100 per cent transmission at 508 mμ. (This is the peak exhibited by most of the compounds studied.) When the sliding exit slit was 5 mm., the entrance slit for light was generally set at about 0.8 mm. The transmission generally was measured from 400 to 550 mμ to establish the transmission curve over the significant part of the visible spectrum. Since several samples were read successively (a special sliding mechanism was employed which will be described in a later paper), the transmissions of the blanks were reset at the original figures to correct for any drift in the machine due to line voltage variations. In this way comparative successive readings could be made. The K value recorded was evaluated from the equation $K = \log I_o/I$, which is numerically equivalent to the optical density calculated from $D = 2 - \log_{10} T$, or $D = 2 - \log I_o/I$. All derivatives were equilibrated to be equivalent to a basic compound.

Zimmermann Test For Ketosteroids

It will be seen in Table I that there is some change in the transmission of light, or shift of the peak, when an OH group, or other groups (as methoxy, benzyloxy), is at position 3. For instance, 3-methyoxyestrone, 3-benzyloxyestrone, and estrone gave K values in that descending order.

Androsterone acetate is about as efficacious as those mentioned above, indicating neither interference of the acetate radical nor need of unsaturation in Ring A for highest color development. In all these cases the carbonyl group is at position 17, and a methylene carbon is at position 16. Estrone-16 differs in that a carbonyl group is now at position 16, with methylene carbons at both positions 15 and 17. No color is obtained with this compound and the Zimmermann reagents. 16-Ketoestrone, with carbonyl groups at both positions 16 and 17 and an adjacent methylene carbon in position 15, develops no color. When an OH or an R group is at position 17 and methylene carbons at both the 15 and 16 positions, no color de-

TABLE I

Structural Configuration of Steroids and Zimmermann and Kober Reagents

Compound	Position occupied	Group at position	Zimmermann reaction*	Kober reaction*
Estrone (1, 3)†	17	Carbonyl	0.278	1.025
Benzyloxyestrone (1, 3)	17	“	0.303	0.871
Methoxyestrone (1, 3)	17	“	0.382	1.115
Androsterone acetate (1, 3)	17	“	0.326	No peak
Equilenin (1, 3)	17	“	0.349	0.807 (490)
Equilin (1, 3)	17	“	0.304	0.494 (470)
Estrone-16 (2, 4)	16	“	No peak	No peak
Methoxyestrone-16 (2, 4)	16	“	“ “	“ “
Benzyloxyestrone-16 (2, 4)	16	“	“ “	“ “
Estriol (1, 2)	16, 17	OH	“ “	0.479 (464); 0.498
Isoestriol-A (1, 2)	16, 17	“	“ “	0.695
α-Estradiol (1, 3)	17	“	“ “	0.532 (436); 0.497
β-Estradiol benzoate (1, 3)	17	17 OH	“ “	0.497
3-Methoxy-16-keto-α-estradiol (1, 2)	16, 17	16 O, 17 OH	“ “	1.056
16-Ketoestrone (1, 2)	16, 17	Carbonyl	“ “	No peak
3-Methoxy-16-ketoestrone (1, 2)	16, 17	“	“ “	“ “
Testosterone (1, 3)	17	17 OH	“ “	0.334 (490)
Cholesterol (1, 3)	17	R	“ “	No peak
Doisynolic acid	Ring D out		“ “	“ “
Marrianolic acid	“ “ “		“ “	“ “

* Peak at 508 $m\mu$ unless otherwise indicated in parentheses.

† Meaning of figures in parentheses in this column as follows: (1) 13 *tert*-carbon; (2) 15 CH_2; (3) 16 CH_2; (4) 17 CH_2.

velops. If Ring D has been broken, as in doisynolic acid and marrianolic acid, no color develops.

Kober Reaction

The above modified Kober reaction was employed with β-naphthol in concentrated H_2SO_4. Table I shows the peak values at 508 mμ as in the Zimmermann reaction. Here again, when the carbonyl group is at position 17 and the methylene carbon is at position 16, the greatest amount of color is produced. However, 3-methoxy-16-keto-α-estradiol is an exception to this. When carbonyl groups are at positions 16 and 17 and a methylene carbon is at position 15 (conditions under which the Zimmermann reaction gives no color), from 10 to 20 per cent absorption of light is noted, but no definite peak is observed.

Results

From a study of the groups affecting the intensity of color development in the Zimmermann and Kober reactions, the following results are reported.

Zimmermann Reaction—(1) Maximum color is developed when the compound contains a carbonyl group at position 17 and a methylene carbon adjacent at position 16 in the perhydro-1, 2-cyclopentanophenanthrene compounds. (2) When a carbonyl group is at position 16 and the two adjacent carbons are methylenic, or when there are carbonyl groups at both positions 16 and 17 and a methylenic carbon at position 15, no color is produced. (3) When an OH or R group is at position 17 with an adjacent methylenic carbon at position 16, no color development is observed. (4) If position 3 has a methoxy or benzyloxy group on it, there is a significant effect on the transmission of light. (5) Unsaturation of Ring A or Rings A and B does not significantly affect the color development.

Modified Kober Reaction—(1) Maximum color is developed when the compound contains a carbonyl group at position 17 and a methylenic carbon at the 16th position of all the estrogens tested. (2) If an OH group is at position 17, or at both positions 16 and 17, together with adjacent methylenic carbons, the K value is roughly 50 per cent of that of compounds with a carbonyl group at the 17th position. (3) Methoxyestrone gives more color but the benzyloxy derivative gives less color than estrone in the Kober test. (4) Carbonyl groups at position 16 or at both the 16th and 17th positions gave no color and hence showed no peak value. (5) A hydroxyl group at position 17 and a carbonyl group at position 16 show as much transmission activity as a carbonyl at position 17 with an adjacent methylenic carbon.

SUMMARY

1. In the Zimmermann reaction, maximum color development depends on a carbonyl group being at position 17 and a methylene carbon at 16. No other group on or modification of Ring D of the perhydro-1, 2-cyclopentanophenanthrene nucleus gives a positive value.

2. In the modified Kober reaction, maximum color development is noted as in the Zimmermann reaction above. Other groups in other positions give definite color development. Especially noteworthy is 3-methoxy-16-keto-α-estradiol which replaces the methylenic carbon at position 16 with a carbonyl group. Equilenin, which has an aromatic Ring B, gives a very high peak. Equilin, which is less unsaturated than equilenin, has a *K* value of approximately one-half that of equilenin.

The author wishes to acknowledge his indebtedness to the following for their contributions to this paper by generously furnishing pure samples for the tests: Research Division, the Schering Corporation, Bloomfield, New Jersey, for α-estradiol, β-estradiol benzoate, testosterone, and androsterone acetate; Research Laboratories, Parke, Davis and Company, Detroit, Michigan, for equilenin, theelin, and theelol; Dr. Ernest J. Umberger, Pharmacologist, Food and Drug Administration, Washington 25, D. C., for equilin; and Dr. Max N. Huffman and coworkers for benzyloxy- and methoxyestrone, 16-ketoestrone, 3-methoxy-16-keto-α-estradiol, estrone-16, isoestriol-A, doisynolic and marrianolic acids. The author also wishes to express his gratitude for the help of his technicians, Mrs. Tom Morgan and Mrs. John Little, for their contribution to the paper.

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THE EFFECTS OF FEEDING EXCESS DL-METHIONINE AND CHOLINE CHLORIDE TO RATS ON A CASEIN DIET*

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It has been shown by du Vigneaud and coworkers (1–3) that the methyl group as it occurs in methionine, choline, and other sources is a dietary essential. These investigators and others have also shown (4–6) that man and other animals are capable of transferring this methyl group from one compound to another, as, for example, from choline to homocysteine to form methionine, and from methionine to guanidoacetic acid to form creatine. This process is known as transmethylation.

By the use of radioactive tracers (7) it has been proved that the methyl group of methionine may also be oxidized directly to carbon dioxide, as much as one-third of that ingested being oxidized by the rat in 52 hours.

The metabolism of methionine and choline also bears a close and important relationship to fat metabolism, since a lack of either of these dietary essentials will lead to the formation of fatty livers and hemorrhagic kidneys (8–9).

A new method of approach has been to feed excessive amounts of methionine, under which conditions certain striking physiological changes may be detected (10–11). It has been shown (12) with Sherman strain rats that the addition of 4.8 per cent methionine to a 12 per cent casein diet causes a marked loss in weight, a large part of which represents fat stores of the animal. The animals may continue to lose weight while in positive nitrogen balance under these conditions, which may be interpreted to mean that extensive catabolism of fat is continuing. At the same time there is a marked hypertrophy of the kidney.

If the effects observed are due to the increased demands of the body to metabolize methyl groups, either by oxidation or by elimination as the methyl group of creatinine, we might expect that methyl groups of choline, fed in excessive amounts, would tend to cause similar physiological changes. The work described below was designed to test the effects of feeding a high level of choline and to explain further the relationships existing between this compound and methionine.

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* This work was done under contract with the Office of Naval Research, Navy Department, Washington, D. C.

EXPERIMENTAL

Five groups of male, Sherman strain rats, weighing approximately 250 gm. each, were utilized. There were ten rats in each group, housed two in a cage. The basal diet consisted of the following ingredients per 1000 gm.: Labco casein 120 gm., sucrose 154 gm., dextrose 222 gm., dextrin 202 gm., lard 252 gm., Wesson's salt mixture (13) 17 gm., and agar 33 gm. To prepare the diet, 1400 gm. of water were utilized per 1000 gm. of solid materials. The agar was first dissolved in two-thirds of the boiling water, and then the lard was added, followed by a slurry of the other ingredients in the remainder of the water. Mechanical stirring was employed to obtain a nearly homogeneous mixture and any losses in water due to evaporation were made up. Just before the diet was ready to solidify, the following vitamin supplement, per kilo of dry diet, was stirred into the mixture: thiamine hydrochloride 2 mg.; pyridoxine hydrochloride 1.6 mg.; calcium pantothenate 40 mg.; niacin 40 mg.; 2-methyl-1,4-naphthoquinone 0.2 mg.; *p*-aminobenzoic acid 40 mg.; inositol 100 mg.; riboflavin 3.2 mg.; biotin 0.2 mg.; folic acid 0.2 mg.; cod liver oil, containing 1800 U. S. P. units of vitamin A and 180 U. S. P. units of vitamin D per gm., 10 gm.; and α-tocopherol 40 mg.

The five groups of rats received the following, in addition to the basic diet, per 1000 gm. of dry ingredients: Group I, 48 gm. (4.8 per cent) of DL-methionine plus 1 gm. of choline chloride; Group II, 70 gm. (7.0 per cent) of DL-methionine plus 1 gm. of choline chloride; Group III, 48 gm. (4.8 per cent) of DL-methionine (no choline chloride); Group IV, 48 gm. (4.8 per cent) of DL-methionine plus 13.5 gm. (1.35 per cent) of choline chloride; and Group V, 13.5 gm. (1.35 per cent) of choline chloride.

The animals in Group I served as controls. The other groups were fed the same weight of diet as was eaten by Group I. Collections of urine and feces were made as previously described (12). The nitrogen intake of Groups I, II, III, and V was approximately the same (Table I). Group IV had a higher intake owing to a higher nitrogen content of the diet. The diet containing 7 per cent DL-methionine (Group II) also had a higher nitrogen content, but the rats on this diet ate less than the controls. In Table II are listed the organ weights of the five groups.

Consideration of the data in Tables I and II shows the following.

The addition of 1.35 per cent choline chloride (Group V) caused no hypertrophy of the kidney, the values for this organ being identical with control values obtained from rats fed the basic diet alone (12). The animals fed excess choline chloride lost considerable weight during the 20 day experimental period, but this also occurs with the basic diet, owing to restriction of food intake. During the last 10 days of the experimental period the animals fed excess choline chloride were in positive nitrogen balance and gained weight slowly. On the other hand, Sherman strain rats fed 4.8 per cent DL-methionine (Group I), although in positive nitrogen balance during the last 10 days, continued to lose weight slowly. The loss represented fat stores, since the water content of the tissues examined did not change and there was a striking lack of fat in the animal carcass.

TABLE I

Effect of Dietary Supplements of DL-Methionine and Choline Chloride on Nitrogen Balance and Body Weight of Rats during 20 Days

There were five cages of animals in each group. Four 4 day collections were made from each cage and analyses performed in duplicate on each collection. The results were averaged to give the values (twenty values obtained on ten rats in each group) in the table.

Group No.	Supplement		Ingested N	Urinary N	Fecal N	Nitrogen balance	Weight change
	Methionine	Choline chloride					
	per cent	*per cent*	*mg. per kg. per day*	*mg. per kg. per day*	*mg. per kg. per day*	*gm. per kg. per day*	*gm.*
I	4.8	0.1	538	423	82	+0.033	−36.6
II*	7.0	0.1	515	668	74	−0.227	−67.0
III	4.8	0.0	495	493	75	−0.073	−52.4
IV	4.8	1.35	638	550	93	−0.005	−43.3
V	0.0	1.35	515	400	106	+0.009	−33.6

* Sixteen values on eight rats.

TABLE II

Effect of Dietary Sources of DL-Methionine and Choline Chloride on Organ Weights of Rats

The values given are average wet weights per 100 gm. of body weight of ten animals in each group.

Group No.	Supplement		Liver	Kidney	Adrenals	Thyroid	Testes	Seminal vesicles
	Methionine	Choline chloride						
	per cent	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*gm.*	*gm.*
I	4.8	0.1	3.15	0.856	12.6	4.5	1.07	0.264
II*	7.0	0.1	2.89	0.900	10.8	4.0	1.15	0.257
III	4.8	0.0	3.04	0.902	14.4	5.1	1.19	0.157
IV	4.8	1.35	3.37	0.860	11.6	4.5	0.89	0.196
V	0.0	1.35	2.87	0.636	11.6	3.9	1.08	0.384

* Average of eight rats.

The absence of choline chloride in the diet containing 4.8 per cent DL-methionine (Group III) aggravated the effects of the methionine. There was a greater loss in weight, a change from a slightly positive to a negative nitrogen balance, and a greater hypertrophy of the kidney. Lack of choline chloride also gave rise to atrophy of the seminal vesicles.

The addition of 1.35 per cent choline chloride to a diet containing 4.8 per cent DL-methionine (Group IV) did not significantly alter the results obtained by feeding 4.8 per cent DL-methionine alone (Group I).

A diet containing 7 per cent DL-methionine (Group II) caused extensive tearing down of the body tissues of the animals, the high negative nitrogen balance continuing at the same levels throughout the 20 day experimental period. Despite this breakdown of body tissue there was a marked hypertrophy of the kidney. There was also an increased excretion of creatine and creatinine under these conditions (Table III).

TABLE III

Creatinine and Creatine Excretion of Rats Fed Excess DL-Methionine

Average excretion for a 20 day period.

Excess methionine fed	Choline fed	Creatinine	Creatine
per cent	*per cent*	*mg. per kg. per day*	*mg. per kg. per day*
7.0	0.1	39.2	6.3
4.8	0.1	28.9	9.4 (12)*
0.0 (Basic diet)	0.1	25.8	5.7 (12)

* The numbers in parentheses refer to bibliographic references.

These last results confirm the reports of Brown and Allison (11) who used rats of the Long-Evans strain. Strain differences are illustrated since the same effects can be obtained in Long-Evans rats with a lower methionine content of the diet. Differences in strains in regard to methionine uptake have also been reported by Rutman and coworkers (14).

DISCUSSION

Excessive quantities of methyl groups, fed in the form of choline chloride, can apparently be metabolized without causing any profound physiological changes in rats. The effects observed when excess DL-methionine is fed may be due, therefore, to the homocysteine portion of the molecule. Du Vigneaud has obtained evidence for this conclusion.[1] The possibility also remains that the intact molecule may be necessary to bring about the observed changes. Hogan (15) has noted that homocystine and choline, when fed together to growing rats, retarded growth to an extent essentially the same as did an equimolar quantity of methionine, but when fed separately they repressed growth only slightly.

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[1] du Vigneaud, V., private communication.

Since it has been demonstrated that glycine will, in part, counteract the effects of excess methionine (12, 15), these effects may be the result of excessive demands of the body for extra glycine, or more likely extra serine which may be formed *in vivo* from glycine (16). This is in agreement with the view that one pathway for the metabolism of homocysteine is its conversion to cysteine by combination of homocysteine with serine to form cystathionine (17–19). When the demand for serine may be very high, as when feeding 7 per cent DL-methionine, actual tearing down of body tissues may take place to meet the requirements, this tearing down of tissue causing an increased creatinine excretion. Significantly, the excretion of creatine is not materially higher when excess methionine is fed up to the level of 4.8 per cent in Sherman strain rats, even when extra glycine and arginine are fed in addition (12), conditions which should be expected to favor the formation of creatinine.

The relationship of the metabolism of methionine to fat metabolism remains obscure, but the continued catabolism of fat and loss of fat stores observed in animals in nitrogen balance on a diet containing 4.8 per cent added methionine would seem to indicate that the catabolism of methionine or homocysteine requires the simultaneous catabolism of fat. The report (20) that large doses of methionine fed to humans gave rise to acetone and β-hydroxybutyric acid ketosis lends credence to this conclusion.

SUMMARY

1. A level of 1.35 per cent choline chloride in a diet containing 12 per cent casein is well tolerated by rats of the Sherman strain, and no unusual physiological changes are observed, as occur when excess methyl groups are fed in the form of methionine.

2. The absence of choline chloride in a diet containing 4.8 per cent DL-methionine aggravates the effects of the excess methionine. In addition, atrophy of the seminal vesicles occurs.

3. A level of 7 per cent DL-methionine fed to Sherman strain rats causes extensive tearing down of body tissue, loss in weight, and increase in the excretion of creatinine. A marked hypertrophy of the kidney is also noted.

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ENZYMATIC SYNTHESIS OF ORNITHURIC ACIDS*

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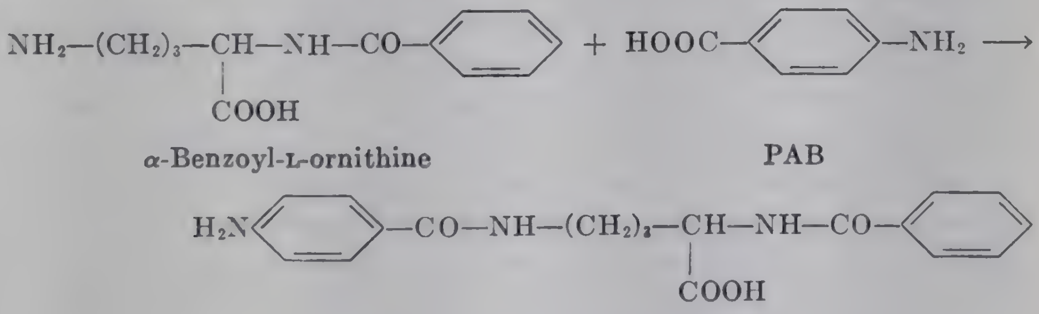
Following the demonstration of the formation of *p*-aminohippuric acid (PAH)[1] from PAB and glycine (1–3) by rat liver, a parallel study of the formation of the *p*-aminoornithuric acids by chicken tissues was undertaken. This was done in order to compare the benzoylation of an optically active amino acid with that of glycine, and with the expectation the system might prove to be free of some of the difficulties besetting the study of PAH synthesis; namely, the low order of anaerobic activity when ATP is the sole source of energy for the reaction, and the *apparent*[2] inability to disrupt the cell particulates without destroying their activity in the synthesis.

In order to separate the benzoylations of the α- and δ-amino groups of ornithine, the α- and δ-monobenzoyl derivatives of L-ornithine were synthesized. These compounds, containing one free amino group, were then used as substrates, together with PAB, to produce enzymatically, as illustrated in Reactions 1 and 2, *p*-aminoornithuric acid or (presumably) *p*′-aminoornithuric acid which could be detected by the analytical method previously employed for PAH.

(1)



α-Benzoyl-L-ornithine

PAB

p′-Amino-L-ornithuric acid

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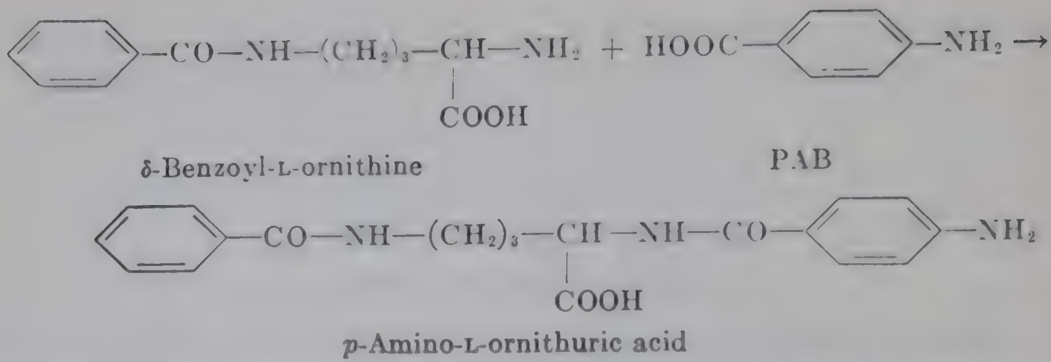
* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

[1] The following abbreviations are used in this paper: PAH for *p*-aminohippuric acid, PAB for *p*-aminobenzoic acid, ATP and ADP for adenosine tri- and diphosphate, respectively.

[2] It has now been shown (R. W. McGilvery, unpublished data) that activity is present in extracts of acetone powders of rat liver and that the loss of activity on treating particulates with water is due in part to a diminished oxidative capacity and in part to an increased ATP-ase activity.

(2)



In exploratory experiments, it was found that chicken liver was devoid of activity toward either substrate, but that the kidney had a low activity toward both compounds. Attention was then concentrated on the α-benzoylation; *i.e.*, on using δ-benzoyl-L-ornithine and PAB as substrates. It was found, as in the synthesis of PAH by rat tissue preparations, that the reaction would proceed anaerobically with ATP as the sole source of energy, and that the activity was associated with the insoluble cellular particulates. Only a survey of the properties of the system was made because of the small extent of the synthesis and because it was evident that further study would be less profitable than with the PAH system.

EXPERIMENTAL

Substrates—L-*Ornithine hydrochloride* (m.p. 232° (decomposition), Cl 21.08 per cent) was prepared from L-arginine by the method of Hunter (4).[3]

α-Benzoyl-L-*ornithine* (m.p. 225–226°, decomposition) was prepared via L-ornithuric acid by the method of Boon and Robson (5).

δ-Benzoyl-L-*ornithine* was prepared by a modification of the method of Neuberger and Sanger (6). 0.02 mole of L-ornithine hydrochloride was dissolved in 20 ml. of water and heated to boiling. Solid basic cupric carbonate was added in small portions until an undissolved excess remained. The excess was centrifuged in a heated tube and then triturated with 4 ml. of boiling water in the tube and recentrifuged. The combined clear, deep blue liquids were then chilled to 0° and 5 N NaOH added dropwise with stirring to about pH 11. The solution was then placed under brisk mechanical stirring in an ice bath and 10 ml. solutions containing 0.021 mole of benzoyl chloride in anhydrous toluene and 0.022 mole of NaOH in water were added in 1 ml. portions at 3 minute intervals. 5 minutes after the last addition, the solution was filtered and the solid, bright blue copper chelate of δ-benzoyl-L-ornithine obtained. The filtrate should have only a faint greenish blue tinge if benzoylation has been complete. The blue solid was thoroughly washed on the funnel, suspended in 70 ml. of water, and heated to boiling. H_2S then was passed into the boiling solution until no more blue particles could be seen. The CuS was filtered and thrice suspended in 15 ml. of boiling water, the suspension being each time resaturated with H_2S and filtered.

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[3] We are indebted to Dr. Santiago Grisolia for an arginase preparation.

The combined filtrates were evaporated on the hot-plate under an air current to a volume of 20 to 30 ml., chilled, and filtered. The resultant δ-benzoyl-L-ornithine (yield 75 to 85 per cent) melted between 257° and 259° when dried. One recrystallization from water usually raised the melting point to 260–261° with small loss, and the melting point was constant with further treatment. Continued evaporation of the mother liquors enabled recovery of a small amount (about 5 per cent) of less pure material melting at 253–255°.

δ-(p-Nitrobenzoyl)-L-ornithine (m.p. 241–242°, decomposition) was prepared in the same manner except that the chelate remained in solution and H_2S was passed directly into the reaction mixture at room temperature, and exhaustive washing of the CuS was not necessary. The yield was much lower (35 to 40 per cent).

p-Nitro-L-ornithuric (m.p. 188–189°, decomposition) and *p′-nitro-L-ornithuric acids* (m.p. 221–224°, decomposition) were prepared by conventional Schotten-Baumann reactions from the above two δ-substituted ornithine derivatives in good yield (85 to 95 per cent).

The corresponding amino compounds were obtained as white crystalline substances in fair to good yields by reduction with ammonium sulfide (7).

The amino compounds were converted to the corresponding *p*-(*p*-hydroxyphenyl)azo derivatives (1) for purposes of comparison. The melting points were as follows: L-α-hydroxy-δ-[*p*-(*p*-hydroxyphenyl)azobenzamido]-valeric acid, darkens at 262°, decomposes at 265°; *p*-(*p*-hydroxyphenyl)azo-L-ornithuric acid, darkens at 222°, decomposes at 224°; *p′*-(*p*-hydroxyphenyl)azo-L-ornithuric acid, darkens at 229°, decomposes at 230–231°. The optical density at 440 mμ of a 0.00001 M solution of each of these compounds was 0.399, 0.406, and 0.409 respectively. The absorption was measured in round cuvettes in the Coleman model 11 spectrophotometer. It is apparent that these derivatives have the expected equivalent weights within 3 per cent. (The agreement is more striking between the latter two compounds in which there is no question of the oxidation of the α-carbon atom by nitrous acid.)

As a further check on the purity of the series of compounds, the amino derivatives were analyzed by the method employed for PAH (1) and the absorption spectra determined in the Coleman instrument. These spectra are shown in Fig. 1 in comparison with that of the azo dye obtained under the same conditions from PAH.

Potassium fumarate was prepared by neutralization of a suspension of the solid acid and precipitation of the salt by stirring a mixture of the hot solution with several volumes of hot alcohol as it cooled. The resultant large clear crystals were washed with alcohol and air-dried, and gave an analysis for ash and water of hydration corresponding to $K_2C_4H_2O_4 \cdot 2H_2O$ within 1.5 per cent for all preparations. This salt is more convenient than the free acid for the preparation of potassium fumarate solutions.

Other substrates have been described previously (1–3).

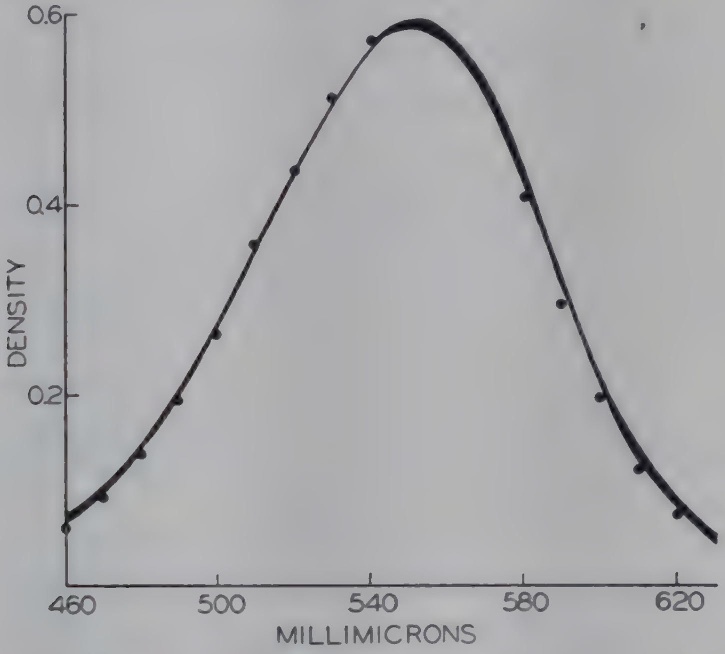

FIG. 1. Absorption spectra of ornithine derivative azo-coupled with *N*-(1-naphthyl)ethylenediamine. The spectra of the azo-coupled derivatives of δ-(*p*-aminobenzoyl)-L-ornithine and *p*-amino- and *p*′-amino-L-ornithuric acids lie within the black areas. Values obtained with PAH falling outside the area are shown as solid points. All were measured at 10 mμ intervals.

Analytical—The analysis was carried out as previously described (2). It was found that 62 per cent of the *p*-amino-L-ornithuric acid was not extracted under the conditions of the analysis. This low recovery introduced further error in the analytical method at low levels of synthesis, as may be seen in the spread of experimental points in the results.

Enzymatic Experiments—KCl homogenates and washed residues of chicken tissues were prepared and incubations were conducted as previously described (2, 3). The hydrosulfite-catalyst mixture was used in the center wells in all anaerobic experiments.

Results

Localization of Enzyme—Homogenates of chicken kidney and liver were tested for activity with both substrates, as were residues and supernatants of the kidney homogenate (Table I). The synthetic activity is localized in the residue fraction. It has been noted previously (3, 8) that this residue contains the nuclei and a variable fraction of the mitochondria of the cell.

Stability of Enzyme—The activity of the entire homogenate toward either substrate is quite stable to storage in the cold (Fig. 2). The similarity of the rates of decline of the two activities might be interpreted as evidence that the same enzyme catalyzes both benzoylations. Actually, in this type of experiment, in which the activity is dependent upon the maintenance of ATP by aerobic phosphorylation, three events will influence the magnitude of the synthesis: changes in the activity of the oxidative systems, of the phosphatases attacking ATP, and of the synthesizing enzyme itself. Data obtained in another connection with the PAH systems lead to the conclusion that the loss of activity of the residue under various conditions, such as storage, water treatment, acetone drying, etc., is due primarily to an interference with the oxidative metabolism or to an increase in the phosphatase activity. This is probably the cause of the decline in the activity exhibited in Fig. 2 and no conclusions about enzyme identity can be drawn.

TABLE I

Localization of Enzyme

Substrate concentrations, 0.001 M PAB, 0.015 M α- or δ-benzoyl-L-ornithine, 0.0025 M $MgSO_4$, 0.001 M ATP, 1.2×10^{-5} M cytochrome *c*, 0.0025 M potassium fumarate. Total volume 4.0 ml., made isotonic with potassium phosphate at pH 7.4. Incubation time, 1 hour. Residue prepared by centrifuging 10 minutes at 2000 × *g* without washing.

	Experiment 1				Experiment 2			
	α-Benzoylornithine		δ-Benzoylornithine		α-Benzoylornithine		δ-Benzoylornithine	
	Liver homogenate	Kidney homogenate	Liver homogenate	Kidney homogenate	Kidney residue	Kidney supernatant	Kidney residue	Kidney supernatant
Tissue nitrogen, *mg*. .	5.97	5.00	5.97	5.00	1.13	1.70	1.13	1.70
Total synthesis, *μM*. .	0.02	0.90	0.00	1.37	0.57	0.00	0.62	0.00

Substrate Concentration—As is shown in Fig. 3, a large excess of the ornithine derivatives is necessary to saturate the system, although this effect is not as pronounced as that with glycine in the PAH synthesis (2). It is possible that the high level of the amino acid acts to inhibit phosphatases as shown by Bodansky (9) and thereby aids in the maintenance of either ATP or phosphorylated cofactors necessary for the oxidation of fumarate. In a similar experiment it was demonstrated that 0.0008 M PAB saturated the system. At this point in the study, work with the α-benzoyl com-

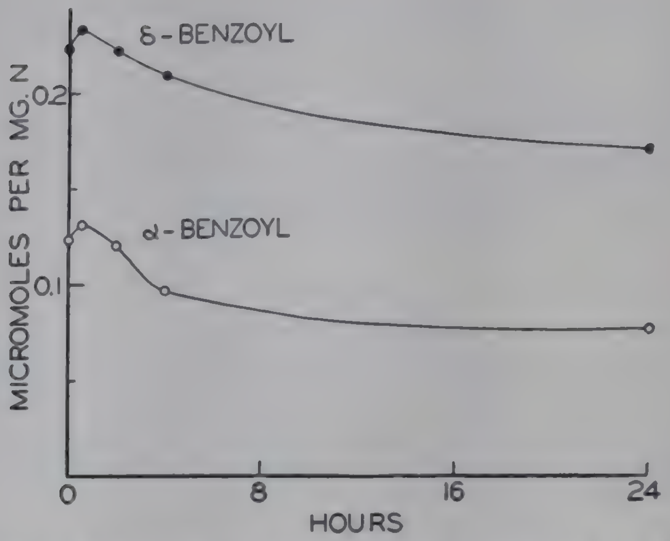

Fig. 2. Stability of KCl homogenate to storage in the cold. A KCl homogenate was placed at 0° and samples were withdrawn at intervals for enzymatic test. The concentrations and conditions were as in Table I. Tissue nitrogen, 4.84 mg. per flask.

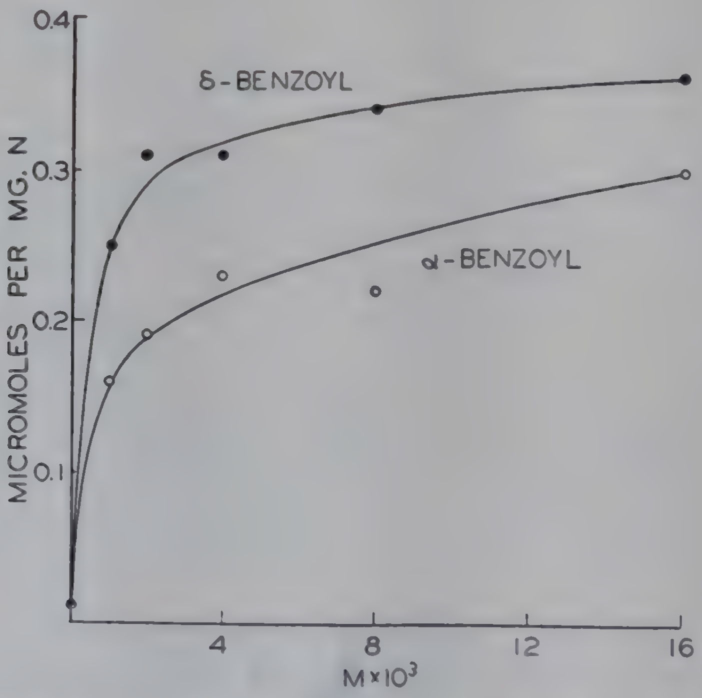

Fig. 3. Effect of α- and δ-benzoyl-L-ornithine concentration on the synthesis of the p-amino-L-ornithuric acids. The concentrations and conditions were as in Table I. Washed residue nitrogen, 1.07 mg. per flask.

pound was discontinued, since it showed less activity and the α-benzoylation was of primary interest.

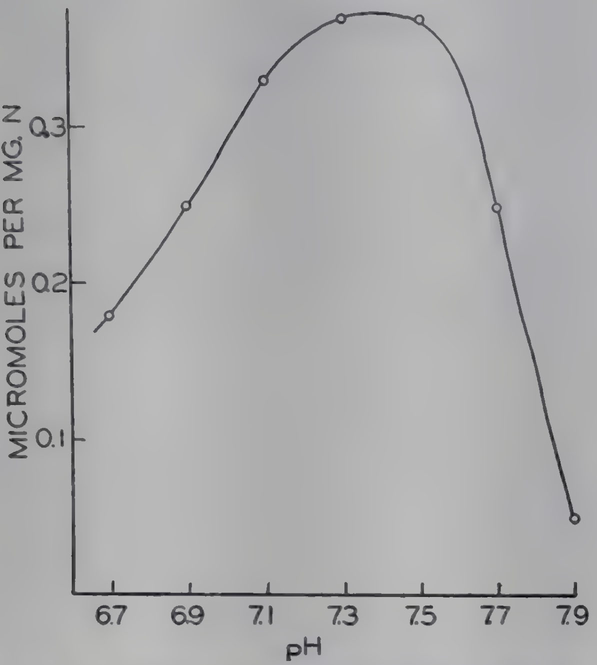

FIG. 4. Effect of pH on *p*-amino-L-ornithuric acid synthesis. The concentrations and conditions were as in Table I. Washed residue nitrogen, 3.20 mg. per flask.

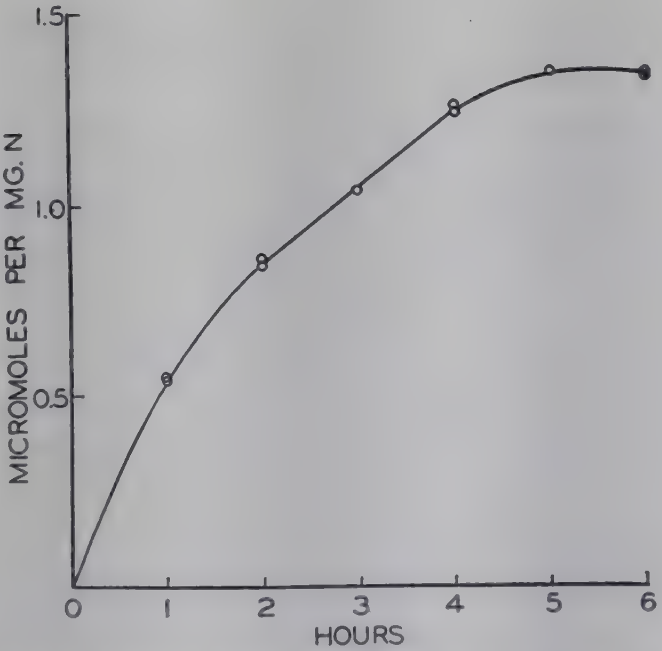

FIG. 5. Synthesis of *p*-amino-L-ornithuric acid as a function of time. The concentrations and conditions were as in Table I except that ATP was 0.002 M, potassium fumarate 0.01 M, and cytochrome *c* was eliminated. Washed residue nitrogen, 2.46 mg. per flask.

pH and Time Curves—The entire system exhibits a pH optimum at 7.4 (Fig. 4). Here again it is to be noted that this is an optimum for *all* of the enzymatic factors affecting the synthesis taken together and not for the synthesizing enzyme *per se*. The system continues synthesis for 5 hours under the conditions of the experiment (Fig. 5), finally achieving over 90 per cent coupling of the PAB available.

Miscellaneous Factors—In the experiments of Table II, it was established that calcium is inhibitory, as is generally the case in systems dependent upon aerobic phosphorylation. Magnesium is required for full activity, and 0.0015 M is adequate to saturate the system. At least 0.01 M fumarate is required for full aerobic activity and no synthesis could be detected in its absence. Unlike the rat liver residue, the chicken preparation does not require added cytochrome *c* for maximum aerobic synthesis.

TABLE II

Effect of Various Additions

Substrate concentrations, 0.001 M PAB, 0.015 M δ-benzoyl-L-ornithine. Total volume 4.0 ml., made isotonic with potassium phosphate at pH 7.4. Incubated 1 hour with air as gas phase. 1.61 mg. of washed residue nitrogen per flask.

Addition	Molarities						
$CaCl_2$	0.00	0.0025					
$MgSO_4$	0.0038	0.0038		0.015	0.0038	0.0038	0.0038
ATP	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Fumarate	0.0025	0.0025	0.0025	0.0025		0.01	0.0025
Cytochrome $c \times 10^5$	1.2	1.2	1.2	1.2	1.2	1.2	
Synthesis per. mg. N, μM	0.28	0.00	0.18	0.28	0.00	0.38	0.30

ATP Requirement—The dependence of the reaction on the presence of ATP is clearly shown under aerobic and anaerobic conditions in Fig. 6. A higher saturation level is required than for the PAH systems (3) in which 0.0005 M ATP is adequate with aerobic conditions, under which its regeneration through oxidative phosphorylation is possible. The small extent of synthesis obtained anaerobically (Fig. 7) is explained by the rapid breakdown of ATP in this type of tissue preparation, and the increase due to higher ATP concentrations results largely from the longer time required to complete its destruction by the phosphatases.

Identification of Product—250 ml. of a medium containing 0.002 M ATP, 0.01 M potassium fumarate, 0.0025 M $MgSO_4$, 0.015 M δ-benzoyl-L-ornithine, 0.001 M PAB, and chicken kidney washed residue containing 156 mg. of nitrogen were incubated 6 hours at 38° in five 250 ml. Erlenmeyer flasks with shaking. 10 gm. of solid trichloroacetic acid were added with stirring and the precipitated protein centrifuged. At this point a total of 42 μM of p-aminoornithuric acid was found in the clear centrifugate. The solution was adjusted to pH 3.8 with solid sodium citrate and extracted three times with 3 volumes of ether and once with 1 volume of benzene. At this point, 27 μM of product and 0.0 μM of PAB remained. The solu-

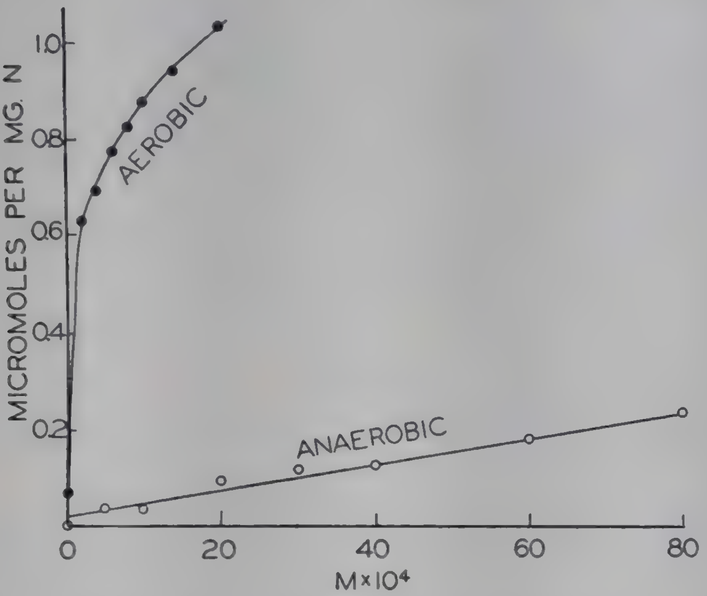

FIG. 6. Effect of ATP on aerobic and anaerobic synthesis of p-amino-L-ornithuric acid. The concentrations and conditions were as in Fig. 5, except that fumarate was omitted in the anaerobic experiment. Washed residue nitrogen, 2.13 mg. per flask.

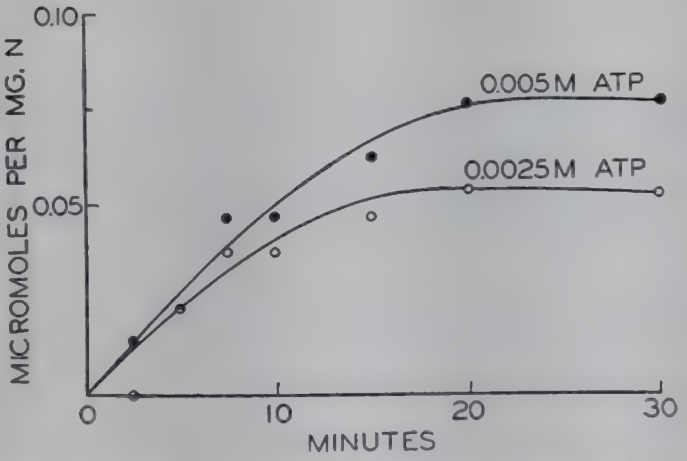

FIG. 7. Anaerobic synthesis of p-amino-L-ornithuric acid as a function of time. The concentrations and conditions were as in Table I, except that fumarate and cytochrome c were omitted and ATP concentrations were as given on the figure. Washed residue nitrogen, 2.18 mg. per flask.

tion was then diazotized, coupled with phenol, and carried through the sequence of ether and bicarbonate extractions as previously described (1). The final twice recrystallized yellow-orange product, 7.3 mg., 15.5 μM, melted at 224° and gave a mixed melting point with *p*-(*p*-hydroxyphenyl)-azo-L-ornithuric acid of 224°. Solutions of the isolated product and known derivative containing 0.0137 and 0.0139 μM per ml. in 0.1 N NaOH were made and the absorption spectra of the two solutions determined in the Beckman instrument with a 1 cm. light path. These spectra, reduced to the absorption of a 0.00001 M solution, are shown in Fig. 8. The anomaly in the curves near 430 mμ occurred owing to the necessity of changes in the slit width.

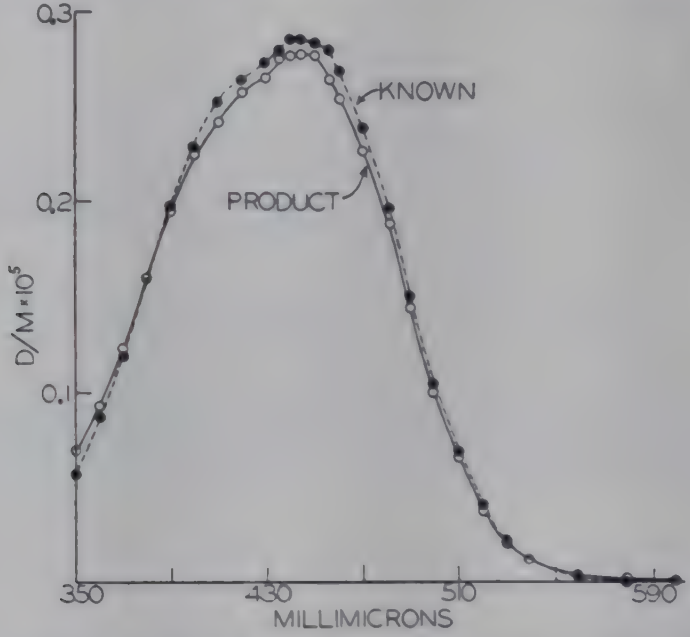

FIG. 8. Absorption spectra of *p*-(*p*-hydroxyphenyl)azo derivative of reaction product and known *p*-(*p*-hydroxyphenyl)azo-L-ornithuric acid in 0.1 N NaOH. The light path was 1 cm. The measurements were made in a Beckman spectrophotometer. Slit width 0.15 mm. to 410 mμ, 0.10 mm. to 430 mμ for the unknown, 435 mμ for the known, and 0.05 mm. above these wave-lengths.

DISCUSSION

At the time the studies on benzoylation were begun in this laboratory, the formation in cell-free preparations of only one other amide, acetylsulfanilamide, had been studied (10). While Lipmann had clearly enunciated the principle that high energy phosphate could be, and is, used in the synthesis of amides, uncertainty still existed in the minds of many investigators. In the brief ensuing period the dependence of not only PAH synthesis, but also the syntheses of such divergent amides as glutamine

(11), citrulline (12), glutathione (13), and now ornithuric acid, on the presence of ATP as an energy donor makes it certain that the view of Lipmann is correct and that the general formulation can be made:

$$R—NH_2 + HOOC—R' + ATP \rightarrow R—NH—CO—R' + ADP + H_3PO_4$$

While this expression represents the over-all reaction, some common intermediate must exist between the amide-forming reaction and the phosphate-hydrolyzing reaction. This intermediate could be, on theoretical grounds, any of the following possibilities (where E is the synthesizing enzyme): $R—NH—PO_3H_2$, $R'—CO—OPO_3H_2$, RNH—ADP, R'—CO—ADP, $E—OPO_3H_2$, E—ADP. Which of these possibilities is the correct one still remains unknown.

SUMMARY

1. The conjugation of PAB with α- and δ-benzoyl-L-ornithine has been studied.

2. The synthesis is accomplished by chicken kidney washed residues, but not by preparations of chicken liver.

3. ATP will support the reaction anaerobically. Under aerobic conditions potassium fumarate addition markedly accelerates the reaction by permitting regeneration of hydrolyzed ATP.

4. *p*-Amino-L-ornithuric acid has been isolated as the *p*-(*p*-hydroxyphenyl)azo derivative.

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THE EFFECT OF ETHIONINE ON TRANSMETHYLATION FROM METHIONINE TO CHOLINE AND CREATINE IN VIVO*

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In order to determine the specificity of the methyl group of methionine in the conversion of methionine to cystine *in vivo*, Dyer (1) tested ethionine (*S*-ethylhomocysteine) to see whether it would replace cystine in the diet. She found that ethionine, in contrast to homocystine and methionine, would not support the growth of young rats on a diet devoid of cystine and containing suboptimal amounts of methionine. It was concluded, therefore, that deethylation of ethionine to yield homocysteine did not occur in the rat. Further evidence in support of this conclusion was presented by Shen and Lewis (2), who found that the rabbit excreted the sulfur of ethionine largely as extra neutral sulfur.

Dyer (1) also observed that supplementation of a 5 per cent casein diet with 0.54 per cent ethionine produced a marked toxicity in rats. The food consumption of animals on the ethionine-containing diet dropped to about two-thirds of that found with the unsupplemented 5 per cent casein diet; animals receiving ethionine lost weight and died after about 10 days, while animals on the unsupplemented diet neither gained nor lost weight. At autopsy, however, no gross pathology due to the toxic effects of ethionine was observed. The toxicity of ethionine could be overcome by including a molar equivalent of methionine in the diet. In this connection, it is of interest that ethionine has been found to inhibit the growth of *Escherichia coli* (3, 4) and *Plasmodium knowlesi* (5), and the growth inhibition for both of these organisms could be reversed by methionine. Although these data do not provide any clue to the mechanism of the action of ethionine, its behavior as a metabolic antagonist may be attributed to interference with some phase of the metabolism of methionine.

Farber, Simpson, and Tarver (6) have reported that ethionine apparently interferes with protein synthesis, since experiments both *in vivo* and *in vitro* showed that ethionine inhibited the incorporation into liver

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* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

protein of S^{35} from labeled methionine and C^{14} from labeled glycine. Experiments *in vivo* with both amino acids indicated that additional methionine would reverse the inhibition. These authors also observed that ethionine inhibited the conversion of methionine to cystine and that this inhibition, also, could be reversed by additional methionine. They suggested that ethionine probably prevented the demethylation of methionine. Stekol and Weiss (7) recently have shown that choline as well as methionine reverses the growth inhibition caused by ethionine in the rat.

The investigation described in the present paper was designed to determine whether ethionine interferes with the transmethylation reactions involved in the formation of choline and creatine. Methionine labeled with deuterium in the methyl group was administered to adult rats and, after a suitable period, the deuterium content of the body choline and creatine was determined as a measure of the extent of transmethylation. Two experiments were carried out, each with a group of four rats. In each experiment, two animals were given ethionine in addition to the labeled methionine, and two animals served as controls, receiving only labeled methionine. Since it was known that the amount of deuteriomethyl transferred from dietary methionine to tissue choline and creatine in a given time is dependent upon the amount of deuteriomethionine ingested (8), the food intake of each group of rats was controlled by the paired feeding technique.

Preliminary feeding experiments to determine the level at which ethionine may be incorporated in the diet without seriously reducing the food consumption of test animals showed that when a basal diet containing 0.7 per cent methionine was supplemented with 0.7 per cent ethionine the daily food intake dropped from over 10 gm. to 1 to 2 gm. per day in 3 days. However, when the ethionine supplement was reduced to 0.5 per cent, the daily food intake dropped only to 7 to 8 gm. The latter diet, in which the molar ratio of ethionine to methionine is about 2:3, was used for the feeding experiments with labeled methionine. In view of the fact that Farber *et al.* (6) have found that ethionine interferes with the conversion of methionine to cystine *in vivo*, it should be noted that the synthetic diet employed in our experiments contained 0.5 per cent cystine.

At the end of the feeding period, the rats were sacrificed, and choline and creatine were isolated from the tissues of each animal and analyzed for deuterium. The data showed that the presence of ethionine in the diet reduced the amount of choline synthesized from dietary methionine by about 20 per cent. On the other hand, the amount of creatine synthesized from dietary methionine was not decreased by the presence of ethionine in the diet.

EXPERIMENTAL

Feeding of DL-*Ethionine and Deuterio*-DL-*methionine*—The four adult male rats in each group were fed *ad libitum* a choline-free amino acid diet for a control period of 8 days, at the end of which time they had regained their initial weight. The percentage composition of this basal diet was as follows: amino acid mixture (9, 10) 21.3, L-cystine 0.5, DL-methionine 0.7, sucrose 53.5, hydrogenated vegetable oil 19, corn oil 1, and Osborne and Mendel's salt mixture (11) 4. The fat-soluble vitamins were included in this diet in the following amounts per 100 gm. of diet: 4000 U. S. P. units of vitamin A, 400 U. S. P. units of vitamin D, 1 mg. of α-tocopherol acetate, 0.1 mg. of 2-methyl-1,4-naphthoquinone. The B vitamins were given twice a day as a supplement. Each rat received daily 20 γ each of thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and nicotinic acid, 0.2 mg. of calcium *dl*-pantothenate, and 25 mg. of choline-free ryzamin-B (9).

TABLE I

Food Consumption and Weight Changes

Group No.	Rat No.	Control period		Experimental period		
		Food intake	Initial and final weight	Diet	Food intake	Final weight
		gm. per day	*gm.*		*gm. per day*	*gm.*
1	1	14.0	260–254	Ethionine	8.1	242
	2	12.4	266–273	"	8.0	254
	3	11.6	270–272	Control	8.0	252
	4	12.9	250–247	"	8.1	232
2	5	11.4	244–246	Ethionine	7.7	235
	6	14.0	236–260	"	7.6	238
	7	10.5	232–239	Control	7.6	230
	8	10.7	253–254	"	7.6	256

During the subsequent experimental period, two rats in each group were given 0.5 per cent DL-ethionine (1), which replaced an equivalent amount of sucrose in the basal diet, and all the rats received in place of the non-isotopic methionine 0.7 per cent deuterio-DL-methionine (12), containing 89.4 atom per cent deuterium in the methyl group.

During the experimental period, the food intake of all rats in each group was limited, by the paired feeding technique, to the amount eaten by the rat with the lowest food consumption. The food consumption and weight changes of the rats during both the control and experimental periods are given in Table I. It will be noted from the data in Table I that the presence of the ethionine in the diet decreased the food consumption from over 10 gm. per day to about 8 gm. per day. With Group 1 the experimental period lasted 9 days, and with Group 2, 14 days.

Creatine was isolated from the carcass of each rat as creatinine potassium picrate (12). Analysis of the recrystallized picrates for creatinine with the Jaffe color reaction indicated that they were pure within the limits of accuracy of the colorimetric method. Choline was isolated from each rat carcass as the choline chloroplatinate (12). The chloroplatinates from Group 1 were recrystallized several times from alcohol-water mixtures, and those from Group 2 were recrystallized three times from water. The platinum determinations for each of the isolated chloroplatinates agreed with the theoretical values.

The creatine and choline derivatives were analyzed for deuterium and the deuterium in the methyl group was calculated on the assumption that all the deuterium in the compounds was present in the methyl group (12). The results are recorded in Table II.

TABLE II

Choline and Creatine Synthesis in Presence and Absence of Dietary Ethionine

Group No.	Rat No.	Diet	Per cent of creatine methyl derived from dietary methionine*	Per cent of choline methyl derived from dietary methionine*
1	1	Ethionine	†	7.20 ± 0.10
	2	"	6.85 ± 0.18	8.66 ± 0.10
	3	Control	5.50 ± 0.18	10.5 ± 0.08
	4	"	5.68 ± 0.18	10.4 ± 0.07
2	5	Ethionine	14.45 ± 0.31	14.65 ± 0.14
	6	"	12.80 ± 0.36	14.82 ± 0.16
	7	Control	12.89 ± 0.36	18.81 ± 0.16
	8	"	12.44 ± 0.36	18.76 ± 0.16

* $\frac{\text{Atom per cent deuterium in methyl groups of isolated compound}}{\text{Atom per cent deuterium in methyl group of methionine fed}} \times 100.$

† Sample lost.

SUMMARY

DL-Ethionine (*S*-ethyl-DL-homocysteine) was fed to adult male rats at the level of 0.5 per cent in a synthetic amino acid diet containing 0.7 per cent deuterio-DL-methionine. The deuteriomethyl content of the tissue choline and creatine was determined and compared with that of choline and creatine isolated from pair-fed controls which received labeled methionine but no ethionine. It was observed that ethionine decreased the amount of transmethylation from methionine to choline by about 20 per cent. The amount of creatine synthesized from dietary methionine was not decreased by the presence of ethionine in the diet.

The authors wish to express their appreciation to Mrs. Josephine T. Marshall for the analyses reported in this paper and to Miss Miriam L. Thompson for her assistance with some of the isolations.

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THE PARTITION RATIO OF ALCOHOL BETWEEN AIR AND WATER, URINE AND BLOOD; ESTIMATION AND IDENTIFICATION OF ALCOHOL IN THESE LIQUIDS FROM ANALYSIS OF AIR EQUILIBRATED WITH THEM

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Accurate information on the partition ratio of alcohol between air and water or body fluids is desirable for several reasons. The values for blood are of fundamental importance in analytical methods in which the alcohol concentration of the breath is employed to predict the level of blood alcohol. A comparison of the figures for blood and urine will shed light on the question whether the level of urinary alcohol represents simple diffusion or true secretion. As pointed out by us in 1939 (1), one may use the air-liquid ratios for water or urine to determine quickly the concentration of alcohol in these fluids from analysis of air equilibrated with them.

Published data for air-liquid partition ratios, covering concentrations of alcohol below about 10 per cent in water or body fluids and temperatures of 0–40°, are few and some of them are quite conflicting. These data, together with our own results reported in this paper, are given in Figs. 5 and 6. The previously published determinations of air-liquid alcohol partition ratios include the following: water at 25° by Foote and Scholes (2), Thomas (3), and Dobson (4); water at 15–26° by Thomas; water at 39.76° by Wrewsky (5); blood at 31–40° by Liljestrand and Linde (6); water, urine, and blood at 10–40° by Haggard and Greenberg (7) in 1934; and water and blood at 20–40° by Haggard *et al.* (8) in 1941. The results for air-water at 25° obtained by Foote and Scholes, Thomas, and Dobson agree quite well, but the ratio for this temperature reported by Haggard and Greenberg is very much higher. Haggard and Greenberg's 1934 figure for air-water at 20° is 3 times that obtained by Thomas, and their 1934 results for air-blood are much higher than those reported by Liljestrand and Linde. With the odd exception of blood at 40°, the 1941 air-liquid figures of Haggard *et al.* are much lower than their 1934 results. However, most of the 1941 figures of Haggard *et al.* remain higher than those of other investigators. Haggard *et al.* ascribe the errors in their 1934 results as being due to loss of alcohol caused by the condensation of water in the cooler parts of their apparatus. This explanation lacks plausibility because moisture condensation would cause *low, not high*, results and also because their greatest errors were at room temperature at which such condensation would be negligible.

During 1929 two of us (B. B. R. and R. N. H.) made a comprehensive study of the air-water alcohol partition ratio for the range of 1–37°, employing alcohol concentrations from 1.0 to 20 mg. per cc. Our results agreed quite well with the few which then appeared in the literature. Shortly afterwards we studied the air-blood alcohol partition ratio. Since Haggard and Greenberg's 1934 paper gave very much higher air-liquid partition ratios, we conducted a large number of additional determinations, with equilibrium approached both from below and from above. These agreed with our earlier results. We made further tests following the publication of the 1941 paper by Haggard *et al.*, but these results simply confirmed our earlier findings.

It should be possible to determine the concentration of alcohol in the water phase from analysis of the air phase, plus the air-water partition ratio for the temperature employed. Here W is the unknown in the equation $k_t = A/W$, where k_t is the Ostwald partition ratio (9) for the temperature used and A and W are the weights of alcohol per unit volume of air and water, respectively. Our experiments showed that this could be accomplished with a satisfactory degree of accuracy, and very rapidly. We found that urine may also be analyzed by this method, by use of the k_t for urine.

Finally, analysis of air equilibrated with the distillate from body materials may be used as confirmatory evidence that a reducing substance in this distillate is all alcohol. This use of partition ratio data is analogous to other physical measurements, such as specific gravity, index of refraction, etc.

EXPERIMENTAL

Partition Ratio of Alcohol for Air-Water, Air-Blood, and Air-Urine

Analytical Method for Alcohol—The alcohol content of both air and liquid phases was determined by the Harger dichromate method (10). Jetter (11) stated that the accuracy of this method is very satisfactory, and the titration procedure for excess dichromate has been employed in a number of other analytical methods for alcohol (12–17). We chose gas volumes and aliquots of solutions or distillates which would consume 70 to 90 per cent of the dichromate. The accuracy of factor W in our calculation formula (10) was also slightly improved by running the acid blank with 0.2 cc. of the dichromate solution and, after titrating, adding a second 0.2 cc. of the dichromate and retitrating. If the first and second titration figures are represented by a and b, respectively, then $W = B + a - b$. B is obtained by a third titration with 1 cc. of the dichromate following titration b. In drying the reaction tubes after they had been thoroughly washed we avoided contact of the open ends with paper toweling or other substances which might permit dissolved organic matter to creep up inside the tube.

Three procedures were used for equilibrating air with the liquid containing alcohol.

Method 1—The apparatus for this procedure is of the conventional type and is shown in Fig. 1. Tubes 1 to 4 each contain 10 to 15 cc. of the alcohol solution being analyzed, and Tubes *a* and *b* each contain 8 cc. of 50 per cent (by volume) sulfuric acid for absorbing alcohol. At first we used a third alcohol absorption tube, but it caught no alcohol. After waiting 30 minutes to permit the bubbler tube contents to reach the bath temperature, air is passed through the tubes at the rate of about 100 cc. per minute. Atmospheric pressure in Tube 4 is maintained by using pressure at the inlet of the train and sufficiently reduced pressure in the air-collecting burette. The volume of air used is measured at room temperature and corrected to the temperature of the bath. 1 cc. of the dichromate solution is then added to Tubes *a* and *b*, the contents are mixed, and the tubes heated in boiling water for 10 minutes. After cooling, the excess dichromate is titrated in the usual way, being stirred by means of a stream of air through the inlet.

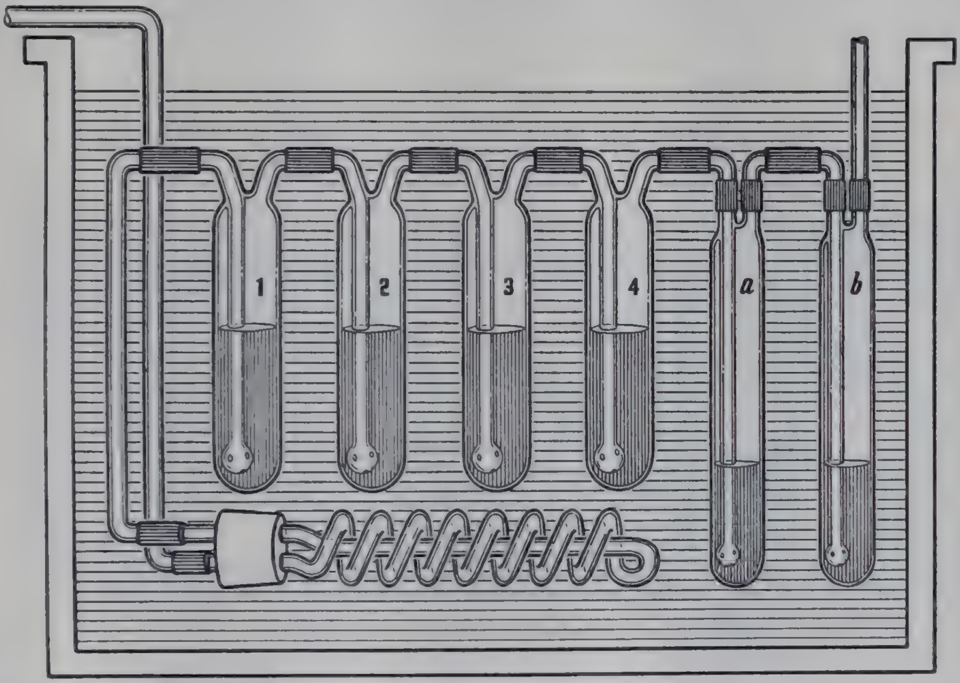

FIG. 1. Bubbler tube train for determining alcohol vapor tension. Tubes 1 to 4 contain the alcohol solution being studied; Tubes *a* and *b*, 50 per cent sulfuric acid for absorbing alcohol from the air which has passed through the four equilibrating tubes.

Method 2—The equilibrating apparatus is shown in Fig. 2 and a portion of the heated exit tube in Fig. 3. The rotating cylinder (*A*) has a capacity of about 500 cc., and during the equilibration period it contains about 100 cc. of the alcohol solution. Bearings for cylinder *A* are provided by the standard taper joint at the left and a ball race at the right. A spring between the ball race and the body of the cylinder prevents leakage at the standard taper joint, which is lubricated with vaseline. Since alcohol is much less soluble in vaseline than in water, the small amount of lubricant used causes no appreciable loss of alcohol. The clamp on the tube leading to the fluid reservoir (*D*) is closed during the half hour equilibration period. At intervals during this period the exit tube (*B*) is raised above the water level and the cap removed to insure atmospheric pressure within the cylinder.

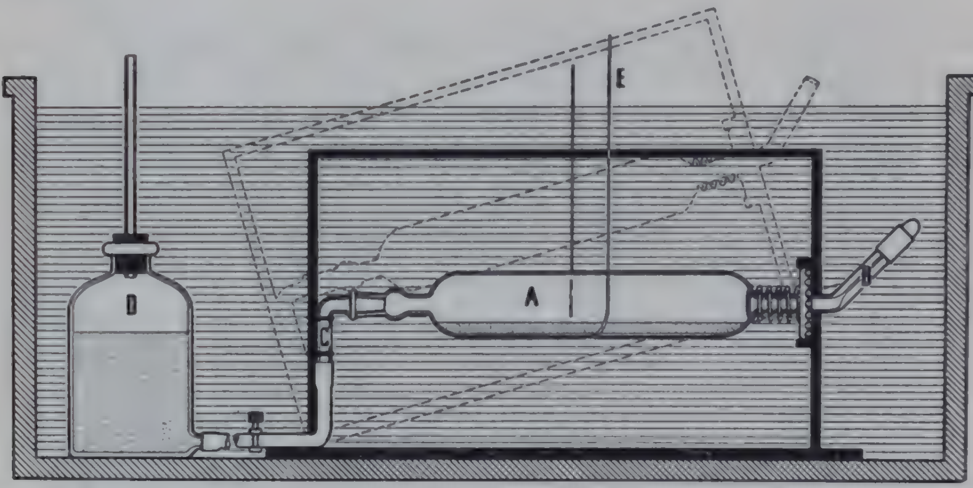

FIG. 2. Rotating cylinder for equilibrating air with blood, water, etc., containing alcohol. *A*, rotating cylinder; *B*, cylinder exit tube; *C*, L-tube for connecting cylinder with reservoir; *D*, reservoir for alcohol solution; *E*, belt.

With this apparatus it is more convenient to have the alcohol absorption portion outside the bath. As pointed out by Thomas (3) and Dobson (4), one must keep the temperature of the exit tube equal to, or higher than, that of the bath. Our exit tube (outside diameter, 0.5 mm.) is heated internally with a piece of 26 gage nichrome wire, as shown in Fig. 3, through which a current of about 0.9 ampere flows, heating the tube to about 45°. The protruding loops of the heating wire prevent moisture condensation at the junctions of this exit tube with the rotating cylinder and the alcohol absorption tube. Experiments with and without the current flowing showed that no alcohol was oxidized at the temperature of the wire. The alcohol absorption tube is of the type shown as Tube *a* in Fig. 1. It is cooled in ice water, and, as shown by tests, a single tube serves to catch all the alcohol. Prior to analyzing the absorption tube contents for alcohol, any moisture plus alcohol which may have condensed just beyond the heated wire in the inlet is washed down with about 0.5 cc. of the 50 per cent sulfuric acid and then by drawing fluid up into the inlet once or twice. The remainder of the analysis is the same as in Method 1.

Method 3—This method is the result of our observation (1) that, if air is discharged through a gas-distributing disk and the tiny bubbles allowed to pass through a 2 inch layer of the alcohol solution, the air attains complete equilibrium in one passage. The apparatus used is shown in Fig. 4.

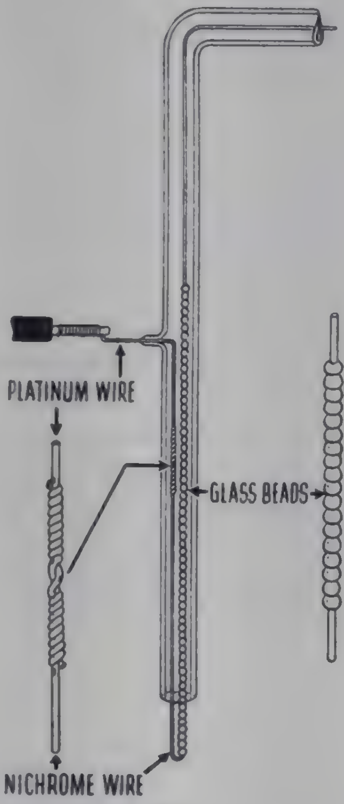

Fig. 3

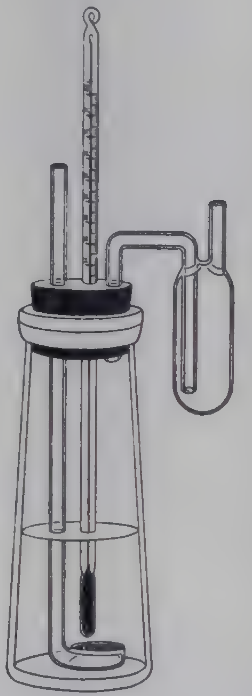

Fig. 4

Fig. 3. Arrangement for internal heating of exit tube and its junction with other apparatus.

Fig. 4. Gas-distributing disk apparatus for equilibrating air with water, urine, or blood containing alcohol.

The cylinder is an 8 ounce urine bottle. A 2 cm. gas-distributing disk (Jena, porosity 1, or Corning, porosity C) is used. The glass trap is connected to the alcohol absorption tube by means of the heated exit tube (Fig. 3). To avoid significant loss of alcohol when the liquid being tested is used for several runs, a second equilibrating apparatus, identical with the first except for the trap, is inserted before the one containing the test solution. The first apparatus contains a water solution of alcohol having a vapor tension of alcohol slightly below, or somewhat above, the test solution. This permits one to approach equilibrium from below, or from above.

In analyzing urine we add 2 or 3 drops of an antifoam fluid which one of us (E. G. B.) developed. It is liquid petrolatum containing 6 per cent of zinc stearate. Tests with water solutions of alcohol showed that the antifoam fluid causes no change in the vapor tension of alcohol. This antifoam material also permits the use of blood in this apparatus. When blood is used, the interior of the bottle is lightly coated with paraffin and the upper parts of the thermometer and gas-distributing tubes are coated with liquid petrolatum. In this way blood may be aerated almost as rapidly as water.

100 cc. of the liquid to be tested are placed in the aeration bottle with the trap outlet, and a like volume of the desired water solution of alcohol in the first bottle. They are connected together and the trap outlet is connected to the heated exit tube. Next the bottles are submerged in the constant temperature bath. When the thermometers in the bottles read the same as the bath thermometer the aeration is conducted, the air being forced through by means of an atomizer bulb. Before connecting the alcohol absorption tube the train and exit tube are flushed out with two or three bulbs full of air. The alcohol absorption tube is the same as that used in Method 2, and is cooled in a beaker of ice water. While an air pressure of about 20 mm. of mercury is required to force the air through each gas-distributing disk, this produces no pressure in the bottle containing the test solution, which is kept at atmospheric pressure by lowering the pressure in the gas burette just sufficiently to balance that required to run gas through the alcohol absorption tube. The remainder of this method is identical with Method 2.

Results

Alcohol Partition Ratio for Air-Water (*A/W*)—The temperatures employed were 1°, 37°, and intervals of 5° from 5–40°. The alcohol concentrations used ranged from 1 to 20 mg. per cc. of water. Method 1 was used for the major portion of the determinations, but a number of them were run by Methods 2 and 3. No differences could be observed in the results by the three methods. For a given temperature two or more concentrations of alcohol were used in order to see whether Henry's law is obeyed in the range of alcohol concentration used. The results indicated that it is obeyed. Thus, at 20° the amount of alcohol per cc. of water and the figure for $k_{A/W} \times 10^3$ were, respectively, 2 mg., 0.157; 5 mg., 0.154; and 10 mg., 0.154. At 30° the corresponding figures were 1 mg., 0.310; 2 mg., 0.311; 5 mg., 0.311; and 10 mg., 0.311. At 37° the figures were 1 mg., 0.472; 2 mg., 0.466; 5 mg., 0.475; and 10 mg., 0.466.

With some of the solutions equilibrium was approached both from below and also from above. The latter was accomplished in Method 1 by placing twice the concentration of alcohol in Tube 1 as in Tubes 2 to 4, with a similar arrangement in Method 3. If the mean of $k_{A/W} \times 10^3$ is represented by $<$ where the equilibrium is approached from below and by $>$ where equilibrium is approached from above, the results obtained for three temperatures were as follows: 25°, $< = 0.217$, $> = 0.219$; 37°, $< = 0.471$, $> = 0.472$; 40°, $< = 0.567$, $> = 0.562$.

The chief experimental details and the results of these determinations are summarized in Table I. In order to conserve space, the figures for individual determinations are not given. The fourth column gives the mean of all determinations for each temperature, and the standard and maximum deviations from this mean appear in the fifth and sixth columns.

TABLE I

Partition Ratio of Alcohol between Air and Water

Temperature	No. of determinations	Concentration of alcohol in water	Mean $k_{A/W} \times 10^3$*	Deviation from mean	
				Standard	Maximum
°C.		*mg. per cc.*		*per cent*	*per cent*
1	6	5-20	0.035	3.5	4.4
5	12	10-20	0.046	4.3	7.1
10	12	10-20	0.073	2.2	3.8
15	12	5-10	0.107	1.6	3.1
20	18	2-10	0.155	2.0	3.9
25	13	2-10	0.217	2.4	3.8
30	23	1- 5	0.310	1.7	3.2
35	6	2	0.418	2.1	3.6
37	28	1-10	0.470	2.2	3.2
40	7	2	0.562	1.8	3.4

* k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/(weight of alcohol per unit volume of water). When the concentration of alcohol in the water is 1 mg. per cc., the value of $k_{A/W} \times 10^3$ equals mg. of alcohol per liter of air.

Fig. 5 gives the curve for the values found by us for $k_{A/W} \times 10^3$ at temperatures of 1-40°. In Fig. 5 we have also plotted the results published by other investigators. Haggard and his colleagues have reported their results as the partition ratio water-air. We have converted them into the corresponding figures for air-water × 10^3. For those publications reporting the partial pressure of alcohol we have translated these figures into mg. of alcohol per liter of air at the temperature used, and then from this and the corresponding water concentration into $k_{A/W} \times 10^3$.

Alcohol Partition Ratio for Air-Blood (*A/B*)—These determinations were run at seven temperatures from 10-40°. Human blood was used in all of the experiments except those run at 40° in which beef blood was used. A determination of hematocrit or the water content was carried out on all blood samples to make sure that they were of normal composition. Data from two blood samples with low solid content are not included in our averages. The anticoagulant used was sodium fluoride in a concentration of about 0.5 per cent in the blood. The alcohol added to the blood varied in strength from 10 per cent in water to absolute. The resulting dilution of the blood was never greater than 2 per cent and was usually much less than this. After adding the alcohol and mixing well, the blood was allowed to stand at least a half hour before being used in order to insure penetration of the alcohol into the corpuscles.

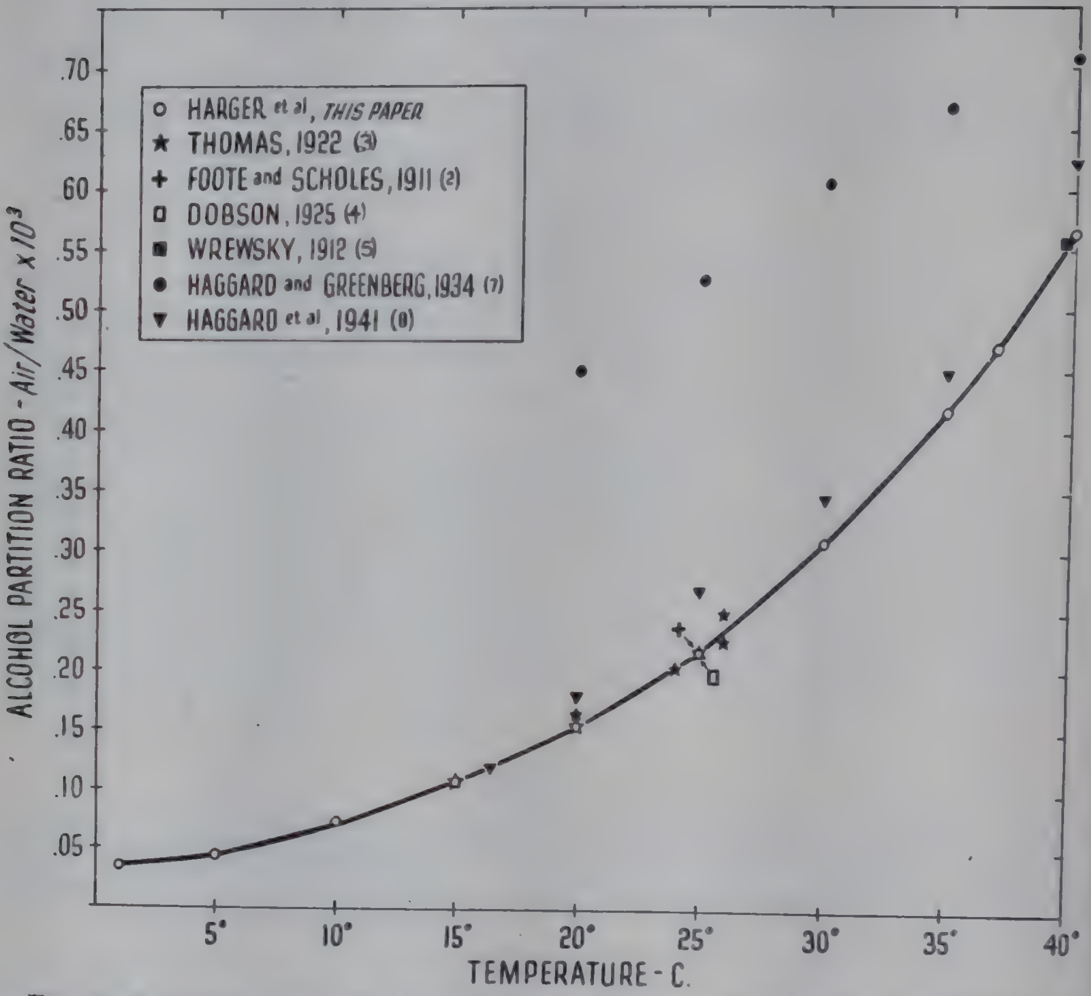

FIG. 5. Partition ratio of alcohol between air and water for temperatures of 1–40°. At 15°, 20°, and 25° our results and those of Thomas are almost identical. At 25° the results of Foote and Scholes and of Dobson are practically the same as that of Thomas and ours.

Method 3 was used for most of the determinations, Method 2 being employed for some of them. The results with the two methods agreed well. Since blood is more viscous than water and might equilibrate with air less readily than water, we approached equilibrium both from below, and from above, with all the blood samples. For each blood sample we ran about the same number of tests from above as from below.

The details and results of our air-blood studies are summarized in Table II. As shown in the next to last column, whether equilibrium was approached from below or from above made very little difference in $k_{A/B}$. The last column in Table II gives the ratio of $k_{A/B}:k_{A/W}$. This ratio was found to be approximately 1.25:1.00 for all temperatures employed. This is not far from the reciprocal of the ratio of water content per unit volume of the two fluids.

TABLE II

Partition Ratio of Alcohol between Air and Blood

Temperature	No. of determinations	Concentration of alcohol in blood	Mean $k_{A/B} \times 10^3$*	Deviation from mean		Change < to >†	$k_{A/B}:k_{A/W}$
				Standard	Maximum		
°C.		*mg. per cc.*		*per cent*	*per cent*	*per cent*	
10	5	8.69	0.092	1.3	2.0	+0.1	1.245
20	7	8.0	0.194	2.6	3.5	+3.0	1.251
30	7	2.0	0.393	2.0	3.1	−0.5	1.263
35	9	2.0	0.515	1.4	2.2	0.0	1.245
37	19	1.0–2.0	0.591	1.7	3.1	+1.5	1.255
40	7	2.0	0.703	1.0	1.6	−1.1	1.250

* k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/(weight of alcohol per unit volume of blood). When the concentration of alcohol in the blood is 1 mg. per cc., the value of $k_{A/B} \times 10^3$ equals mg. of alcohol per liter of air.

† $<$ = $k_{A/B}$ with equilibrium approached from below. $>$ = $k_{A/B}$ with equilibrium approached from above.

A sample of sheep blood having a hematocrit[1] of 38 and a plasma protein content of 5.8 per cent, when run at 37°, was found to have a value of $k_{A/B} \times 10^3$ of 0.547 for the whole blood and 0.509 for the plasma. These results are not included in Table II.

The curve showing our values for $k_{A/B} \times 10^3$ at the various temperatures used is given in Fig. 6. In this chart we have also plotted the figures for $k_{A/B} \times 10^3$ appearing in the literature.

Alcohol Partition Ratio for Air-Urine (A/U)—Equilibration Method 3 was used for urine. Determinations were made at five temperatures from 10–37°. Table III summarizes the data for these experiments. As shown in the last column, the ratio for $k_{A/U}:k_{A/W}$ varied from 1.08:1.00 to 1.09:1.00.

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[1] Per cent of total blood volume occupied by cells.

A sample of urine from a case of diabetes insipidus, having a specific gravity of 1.005, gave 0.474 as $k_{A/U} \times 10^3$ at 37°. This is close to the value of $k_{A/W} \times 10^3$ at 37°, which we found to be 0.470. The results for this urine are not included in Table III.

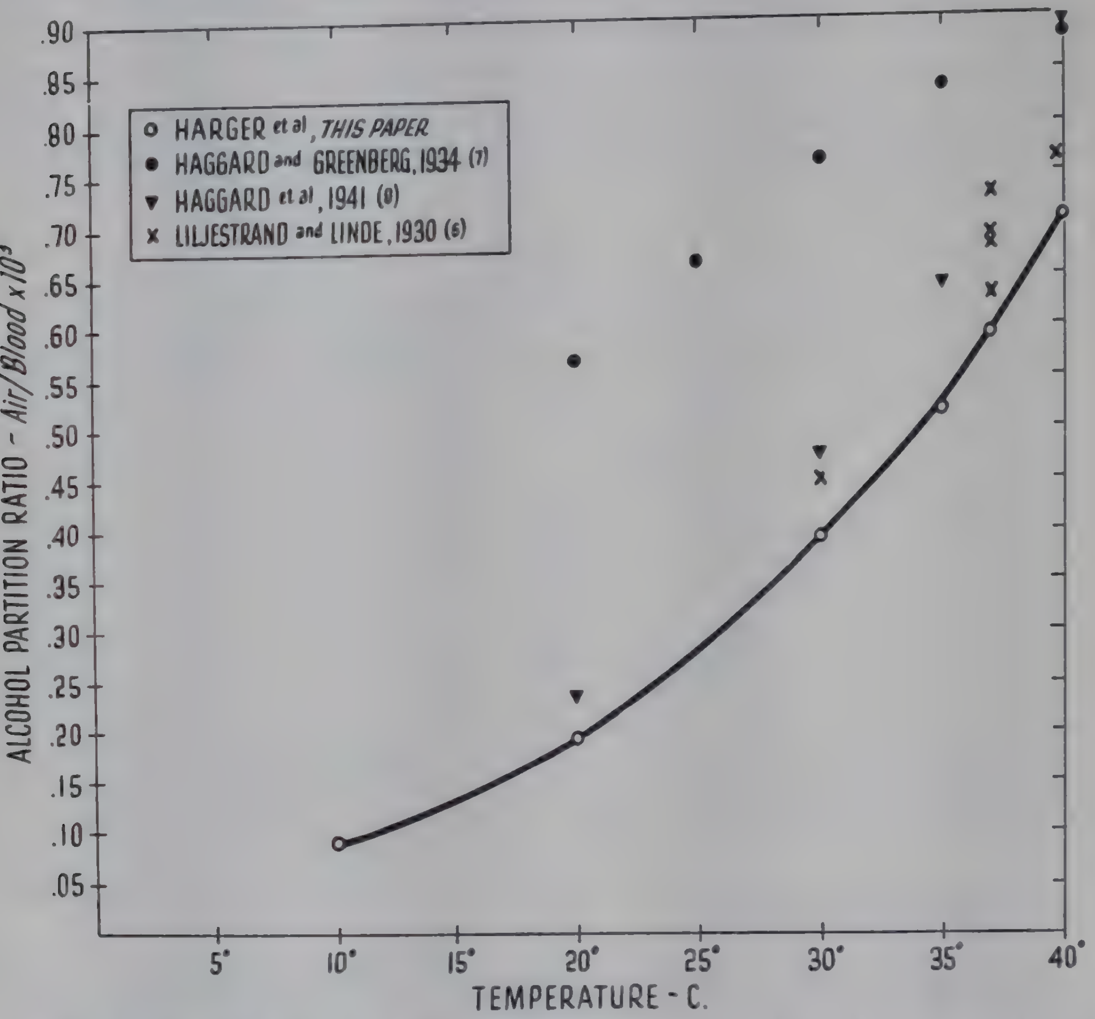

Fig. 6. Partition ratio of alcohol between air and blood for temperatures of 10–40°.

Aerometric Method for Determining Alcohol in Water, Urine, Etc.

Apparatus and Reagents—The fluid to be analyzed is brought to room temperature and equilibrated with air in the apparatus shown in Fig. 4 and the air leaving the trap is analyzed for its alcohol content. The air analysis may be done by the dichromate method mentioned above. However, it was found that the permanganate method of Harger, Lamb, and Hulpieu (18) gives satisfactory results, and, since it uses a smaller weight of alcohol and requires only about 2 minutes to run, it was chosen for this aeration procedure. Only reaction Tube B which is shown in Fig. 4 of this 1938 paper (18) is used.

Following the publication of this permanganate method (18) we have added a color-matching tube, placed next to the reaction tube, in order to read the end-point of the reaction more accurately. The matching fluid is made by mixing 27 cc. of 5 per cent cobalt sulfate solution,[2] 1 cc. of 1 per cent potassium dichromate, and 89 cc. of water. Since this matching fluid contains a trace of pink color, which adds to the precision of reading, a very small amount of permanganate remains at the end-point. This results in a slightly lower alcohol equivalent for 1 cc. of 0.05 N $KMnO_4$, which is 0.169 mg. of alcohol instead of 0.175 as given in our original paper. The figure for the alcohol equivalent of the permanganate was determined by titration with a solution of alcohol in the same concentration of sulfuric acid containing 0.2 mg. of alcohol per cc. The illumination of the reaction tube and matching tube should be from the rear and not from the front or side.

TABLE III

Partition Ratio of Alcohol between Air and Urine

Temperature	No. of determinations	Concentration of alcohol in urine	Mean $k_{A/U} \times 10^3$*	Deviation from mean		Change < to >†	$k_{A/U}$: $k_{A/W}$
				Standard	Maximum		
°C.		*mg. per cc.*		*per cent*	*per cent*	*per cent*	
10	5	8.0	0.080	2.1	2.5	−1.6	1.09
20	8	4.0	0.169	1.4	2.9		1.09
30	14	2.0–4.0	0.338	1.6	2.1	0.0	1.09
37	21	1.64–2.0	0.509	2.3	3.4		1.08

* k, the Ostwald partition ratio, = (weight of alcohol per unit volume of air)/(weight of alcohol per unit volume of urine). When the concentration of alcohol in water is 1 mg. per cc., the value of $k_{A/U} \times 10^3$ equals mg. of alcohol per liter of air.

† $<$ = $k_{A/U}$ with equilibrium approached from below. $>$ = $k_{A/U}$ with equilibrium approached from above.

No constant temperature bath is needed in this method of analysis because it is conducted at room temperature and this will not change appreciably during the period of about 2 minutes required for running the analysis. To facilitate cleaning and drying the trap and its connections between runs, a trap closed with a 2-hole rubber stopper is substituted for the all-glass one shown in Fig. 4.

Procedure

100 cc. of the alcohol solution are placed in the equilibrating bottle (Fig. 4). With urine, 2 or 3 drops of the antifoam liquid are added. The

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[2] 5 gm. of $CoSO_4 \cdot 7H_2O$ are dissolved in 100 cc. of water and then 0.5 cc. of 50 per cent sulfuric acid added.

fluid is brought to room temperature by cooling, or warming, the bottle with water. After flushing out the bottle and trap with one or two bulbs full of air the trap outlet is connected to the reaction tube and this in turn to the gas burette. With a slightly reduced pressure in the gas burette, air is forced through the apparatus at the rate of 200 to 500 cc. per minute until about four-fifths of the permanganate color has disappeared. The remainder of the test is conducted slowly, running the air through intermittently in spurts of 10 to 20 cc. and waiting about 5 seconds between spurts of air. The air passage is stopped at the point at which the color in the reaction tube reaches that of the matching fluid or is just perceptibly lighter. The temperature of the fluid being tested is read with an accuracy of 0.1°. No correction in gas volume is needed, since the temperatures are identical in the equilibrating bottle and the gas burette.

For greater accuracy one may conduct one or two more determinations with the same alcohol solution. The contents of the reaction tube are drained and new reagents added without rinsing the tube with distilled water unless the end-point is distinctly passed in the previous test.

When solutions in the equilibration bottle are changed, the stopper and upper portion of the glass parts should be wiped dry. Moisture here will change the alcohol content of the air which has passed through the fluid. Also, the trap and its glass connections and the rubber tube connecting it to the reaction tube must remain dry. Although the lower portion of the bubbler inlet in the reaction tube is wet with acid, this will cause no error if the level of acid in this tube reaches the same point between intermittent passages of air through the apparatus.

Tests showed that so long as the last part of the passage of air is conducted slowly the time for completing the analysis is not important. Determinations conducted when a period of 5 minutes elapsed between the consumption of most of the permanganate and the completion of the test yielded the same results as tests completed in 2 minutes from the start.

Calculation

Water Solutions of Alcohol—The weight of alcohol required to decolorize the permanganate is constant, 0.169 mg., the variable in this analysis being the volume of the equilibrated air containing this weight of alcohol. This is the volume $V_{\text{obs.}}$ read in the gas burette. It is inversely proportional to the concentration of alcohol in the air, and also in the water. Next, one calculates the volume of air (V_1) which would have been required at this temperature if the water contained 1 mg. of alcohol per cc. The formula for this calculation is

$$V_1 = \frac{0.169 \times 1000}{k_{A/W} \times 10^3}$$

the result being in cc. The value of $k_{A/W} \times 10^3$ is obtained from the fourth column of Table I or the curve of Fig. 5. Then, $V_1/V_{\text{obs.}}$ = mg. of alcohol per cc. of the aqueous solution of alcohol analyzed. A table giving values of V_1 for 0.1° intervals from about 15–35° is convenient if one has many analyses to make.

Urine—The calculation is the same as for water, except that the value $k_{A/U}$ replaces $k_{A/W}$. An alternative method is to use the calculation for water and divide the result by 1.09, which is the average ratio of $k_{A/U}:k_{A/W}$.

Results Obtained with Method

Twenty water solutions of alcohol, with concentrations ranging from 0.91 to 4.72 mg. per cc., were submitted to an assistant who analyzed them by this method. His average error was ±2.2 per cent, with maximum errors of +5.1 and −5.8 per cent. He also analyzed sixteen urine samples containing 0.49 to 3.35 mg. of alcohol per cc., running duplicate analyses with each sample. The average error for the thirty-two determinations was ±4.0 per cent, with maximum errors of +7.1 and −9.0 per cent. The composition of all samples was unknown to the analyst.

Use of Aerometric-Permanganate Analysis Plus Dichromate Reduction As Identification Test for Ethyl Alcohol

The fluid or tissue being analyzed for alcohol is distilled as usual. A portion of the distillate is analyzed for alcohol by the dichromate method used in the first part of this study. Assuming that the only reducing substance present in the distillate is ethyl alcohol, its concentration in the distillate is calculated and the result expressed as mg. of ethyl alcohol per cc., which we will designate as $C_{K_2Cr_2O_7}$. A second portion of the distillate is run by the aerometric-permanganate method described in the preceding section, with a period of about 3 minutes to reach the end-point. This result is also calculated as the concentration of ethyl alcohol expressed as mg. per cc., which we will call $C_{\text{aer.-KMnO}_4}$. If the reducing material present in the distillate is all ethyl alcohol, $C_{K_2Cr_2O_7}$ will equal $C_{\text{aer.-KMnO}_4}$. If the two are unequal, a part, or all, of the reducing substance is not ethyl alcohol.

This double analysis procedure was applied to a number of aliphatic alcohols and to ether and acetone. The results obtained for the $C_{K_2Cr_2O_7}:C_{\text{aer.-KMnO}_4}$ ratio are given in Table IV. Table IV also includes our results for the dichromate equivalent of the different compounds. With all of the alcohols tested, except ethyl alcohol, the result obtained with the aerometric-permanganate analysis varied somewhat with the time required to reach the end-point, smaller volumes of air (*i.e.* less alcohol) being required if the time interval of analysis was 5 to 10 minutes instead of the usual time of 3 minutes. Methyl alcohol in water has about the same $k_{A/W}$ as ethyl alcohol. The high $C_{K_2Cr_2O_7}:C_{aer.-KMnO_4}$ ratio obtained with methyl alcohol is due to differences in its oxidation with dichromate and with permanganate. In the dichromate reaction ethyl alcohol is oxidized to acetic acid, while methyl alcohol goes to carbon dioxide and water (10). In the reaction with permanganate in the presence of 16 N sulfuric acid our evidence indicates that ethyl alcohol still goes to acetic acid, while methyl alcohol is probably oxidized to formaldehyde. This would mean that in the dichromate reaction methyl alcohol consumes 3 times as much oxygen as in the permanganate reaction. We have found that both ethyl alcohol and acetaldehyde rapidly reduce the permanganate reagent, while acetic acid causes no reduction whatever. In a large scale experiment it was shown that no carbon dioxide was produced. On account of the great excess of sulfuric acid and the presence of intermediate oxides of manganese (18) we did not attempt to identify acetic acid in the solution resulting from the reaction.

TABLE IV

Comparison of Results from Dichromate Analysis with Those from Aerometric-Permanganate Analysis for Certain Aliphatic Alcohols, Ether, and Acetone

Alcohol	Alcohol equivalent of 1 cc. of 0.0434 N $K_2Cr_2O_7$	Ratio, $\frac{C_{K_2Cr_2O_7}}{C_{aer.-KMnO_4}}$ *
	mg.	
Ethyl	0.500	1.00
Methyl	0.230	2.55
Propyl	0.493	0.90
Isopropyl	1.047	0.63
n-Butyl	0.762	0.59
Isobutyl	0.584	0.73
n-Amyl	0.746	0.32
Isoamyl	0.678	0.62
Tertiary amyl	0.722	0.55
Ether	0.403	0.013
Acetone	16.6	No reduction of permanganate reagent

* $C_{K_2Cr_2O_7}$ = dichromate result calculated as ethyl alcohol. $C_{aer.-KMnO_4}$ = aerometric-permanganate result calculated as ethyl alcohol.

With the other alcohols studied the variation of the $C_{K_2Cr_2O_7}:C_{aer.-KMnO_4}$ from that of ethyl alcohol is largely due to the fact that these alcohols have a higher $k_{A/W}$ than that of ethyl alcohol. This is also particularly true with ether, the $k_{A/W}$ of which is more than 100 times that of ethyl alcohol.

DISCUSSION

Larger scale operations for our determinations of the air-liquid alcohol partition ratio would probably have yielded slightly more precise results. However, we believe that any errors in the results obtained are not greater than 1 or 2 per cent.

While the alcohol partition ratio for air-water is constant for a given temperature, the ratios for air-blood and air-urine are subject to some fluctuations, which are approximately inversely proportional to the water content of these fluids (19). This is demonstrated by our results with sheep blood having somewhat low hematocrit and plasma protein values and also with diabetes insipidus urine. Air equilibrated with blood is really a three phase system, air, plasma, and cells, the three having the same alcohol vapor tension. Kunkele (20) obtained serum-clot alcohol ratios varying from 1.07:1.00 to 1.15:1.00, averaging 1.11:1.00 (weight-volume). A few analyses by us gave plasma-whole blood alcohol ratios ranging from 0.93:1.00 to 1.17:1.00 and averaging 1.05:1.00. These results would indicate that bloods with an abnormal cell volume might vary from normal bloods in their $k_{A/B}$ values as much as 5 to 10 per cent. Since variations in the alcohol vapor tension of bloods having the same concentration of alcohol would probably change the alcohol concentrations of breath and brain equally, it would appear that, for individuals with an abnormal water content of blood, breath analysis might predict the level of brain alcohol better than would analysis of blood.

Further evidence that the revised air-liquid alcohol partition ratio figures reported by Haggard *et al.* in 1941 (8) are still incorrect, at least in part, is shown by the variations they found in the relative values for $k_{A/B}:k_{A/W}$. According to their data these vary from 1.29:1.00 at 25° to 1.44:1.00 at 35° and 40°. The factors which cause the vapor tension of alcohol in blood to be higher than that in water should not change 12 per cent in a temperature interval of 10°. Also, we can see no theoretical reason why the alcohol vapor tension of normal blood should ever be 44 per cent greater than that of water having the same concentration of alcohol.

The $k_{A/B}$ ratios reported by Liljestrand and Linde (6) average about midway between ours and those reported by Haggard *et al.* in 1941 (8). If Liljestrand and Linde used normal bloods, the variation in their four figures for 37° indicates some rather larger errors.

For predicting the blood alcohol level from breath analyses the $k_{A/B}$ at 34° is important. As pointed out by Winslow, Herrington, and Nelbach (21), studies by various investigators agree that the average temperature of expired air is 34°. Liljestrand and Linde (6) stated that the temperature at which alveolar air leaves the mouth will control the partition ratio of alcohol between breath and body fluid. Our determinations showed that at 34° $k_{A/B} \times 10^3$ is 0.493, which gives a blood-air ratio close to 2000:1. Simultaneous analyses of human breath and blood reported by Liljestrand and Linde (6), Harger, Lamb, and Hulpieu (18), Jetter, Moore, and Forrester (15, 22), and Fabre and Leheuzey (23) all point to a blood-alveolar air ratio which approximates 2000:1 and is not in agreement with the ratios given by Haggard and colleagues which were 1150:1 in 1934 (7) and 1300:1 in 1941 (8). This matter will be more fully discussed in a later paper.

In the use of our aerometric-permanganate method, smaller volumes of the alcohol solution may be employed by decreasing the diameter of the fluid container. With a cylinder having a diameter of about 4 cm., a 30 cc. sample is sufficient, and by employing an Allihn tube with fritted disk[3] as little as 10 cc. of the alcohol solution may be used. However, when analyzing 10 cc. samples one should correct the result obtained for the drop in dissolved alcohol during the aeration.

SUMMARY

1. We report results of determinations of the partition ratio of alcohol for air-water (A/W) at 1–40°, air-blood (A/B) at 10–40°, and air-urine (A/U) at 10–37°. Three methods of equilibration were used and equilibrium was approached both from above and from below. The range of alcohol concentration used was from 1 to 20 mg. per cc. of liquid, in which range Henry's law was found to be obeyed.

2. At each of the temperatures used the relative values of the air-liquid partition ratios, with $k_{A/W}$ expressed as unity, were 1.25 for $k_{A/B}$ and 1.09 for $k_{A/U}$.

3. Our results for $k_{A/W}$ for alcohol are in substantial agreement with those of Foote and Scholes, Wrewsky, Thomas, and Dobson, but they are definitely lower than most of the 1941 revised figures of Haggard *et al.*

4. Our results for $k_{A/B}$ for alcohol are much lower than the 1941 figures of Haggard *et al.* and somewhat lower than those of Liljestrand and Linde.

5. At 34°, the temperature at which alveolar air is exhaled, we found that $k_{A/B} \times 10^3$ is 0.493, which would indicate a ratio of about 2000:1 for alcohol concentrations in blood to expired alveolar air.

6. A method is described for the rapid analysis of air equilibrated with a solution of alcohol, from which one may employ the partition ratio at that temperature to calculate the concentration of alcohol in the solution.

7. It was shown that a comparison of the result from dichromate oxidation with that obtained by an aerometric-permanganate analysis will serve to differentiate ethyl alcohol from many other reducing substances.

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[3] Corning No. 37730-30M with lower tube bent into a U.

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STUDIES ON THE METABOLISM OF THIOUREA

I. DISTRIBUTION AND EXCRETION IN THE RAT OF THIOUREA LABELED WITH RADIOACTIVE SULFUR*

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The goitrogenic action of thiourea and its derivatives was first described in 1943 (1, 2). It is known that this series of drugs, while not affecting the uptake of iodide ion by the thyroid gland, interferes with the formation of organic iodine (3–5). The mechanism of this action is unknown. Williams, using a chemical method of analysis, found a significant concentration of thiouracils in the thyroid and pituitary glands (6, 7). A new method of study of this problem was afforded by the preparation of thiourea labeled with S^{35}.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were fed a diet of Purina laboratory chow containing 5 parts per million of iodine. 4 to 12 week-old animals were used to minimize the toxicity of thiourea (8, 9). The thiourea used in the earlier experiments had a specific activity of 12,000 to 28,000 counts per second per mg. in the counters used. In small organs with little evidence of concentration, the counts obtained with this material were so low (0.1 to 0.3 counts per second) that the results were in doubt. Therefore, when material of much higher specific activity (1,500,000 counts per second per mg.) was obtained, some of the experiments were repeated with this material.

Each animal received 1 mg. of thiourea in 1 ml. of saline intraperitoneally and was placed in a metabolism cage. Urine and feces were collected. Expired air was passed through three traps containing 0.1 N hydrochloric acid, 0.1 N sodium hydroxide, and 3 per cent hydrogen peroxide successively. The rats were killed with ether at intervals of 6, 24, 48, and 72 hours following injection. Samples included the intact thyroid

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and pituitary glands, portions of other tissues ranging in wet weight from 0.2 to 0.6 gm., and aliquots of urine and dried feces. After tissue samples were collected, the remainder of the body was dissolved in an ashing mixture and an aliquot was taken for ashing. 14 mg. of sodium sulfate were added to each sample as a carrier before ashing. Ashing was carried out by the method of Boursnell *et al.* (10). The ash was dissolved in dilute hydrochloric acid, transferred to a 40 ml. centrifuge cone, precipitated with 5 ml. of 5 per cent barium chloride solution, and heated in an oven at 90° for 1 hour. The precipitate was centrifuged, washed twice with water and once with 95 per cent ethyl alcohol, and plated on a copper disk according to the method of Hendricks *et al.* (11). The samples were counted with a mica window Geiger-Müller counter. A correction for thickness was applied by use of an experimentally determined absorption curve.

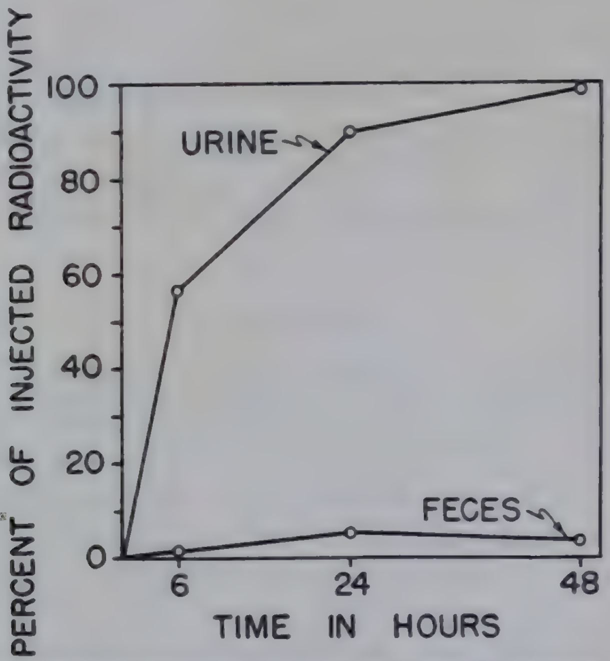

Fig. 1. Excretion of radioactivity in urine and feces following injection of labeled thiourea.

To determine the distribution of the sulfide ion when injected intraperitoneally, a group of six rats was injected with an aqueous solution of barium sulfide labeled with S^{35} containing an undetermined quantity of carrier. Similarly, the distribution of the sulfate ion was determined by injecting rats with a saline solution of carrier-free sulfuric acid labeled with S^{35}. The rats were killed 24 hours after injection and the radioactivity of the thyroid glands was determined as above.

Urine inorganic sulfate and ethereal sulfate were separated chemically according to Folin's method (12). The radioactive urinary components were also studied by one-dimensional filter paper partition chromatography. 0.05 ml. samples of urine were placed on 60 × 2 cm. strips of Whatman No. 1 filter paper and chromatograms were developed in the following systems: (1) butanol and water; (2) butanol and 1 N hydrochloric acid; and (3) butanol and 3 per cent ammonium hydroxide. Control chromatograms were developed in the same systems, with radioactive thiourea in water and in experimental urine. After development, radioautographs were made on 14 × 17 inch Blue Brand x-ray film.

FIG. 2. Radioautograph of one-dimensional filter paper chromatogram of urine from a rat injected with radioactive thiourea. Test solutions were placed on each filter paper strip at the dotted line. Strip *A* contained experimental urine, *B* contained experimental urine to which radioactive thiourea was added, and *C* contained radioactive thiourea in water.

Results

The excretion of radioactivity after the injection of S^{35}-labeled thiourea is shown in Fig. 1. Over 98 per cent of the activity was excreted in the urine within 48 hours. Fecal excretion was slight and may have been due in part to contamination with urine. No radioactivity was present in the expired air.

Chromatograms of the urine showed a single major band, the migration of which was similar to that of thiourea. This indicates that the major portion of thiourea was excreted unchanged. Fig. 2 shows the radioautograph of a typical chromatogram. Chemical analysis showed that 6.2 per cent of the radioactivity in the urine was present as inorganic sulfate and 5.9 per cent as ethereal sulfate.

Table I shows the distribution of radioactivity after the administration of labeled thiourea. Each value represents the average of determinations on three animals unless otherwise noted. The results are expressed as the per cent dose per gm. of tissue. The carcass value represents the average concentration in the body. The thyroid and kidney values are significantly greater than that of the carcass. The maximum value for the thyroid gland was reached at 6 hours.

TABLE I

Concentration of Radioactivity in Rats Injected with Radioactive Thiourea

Tissue	Tissue concentration, per cent dose per gm.			
	6 hrs.	24 hrs.	48 hrs.	72 hrs.
Carcass	0.288* ± 0.045	0.0707† ± 0.0133	0.0276‡ ± 0.00206	0.0285§ ± 0.0117
Thyroid	2.99* ± 0.35	2.20† ± 0.41	1.44‡ ± 0.14	1.278 ± 0.233
Kidney.....	0.396 ± 0.138	0.140 ± 0.020	0.113 ± 0.033	
Adrenal....	0.241 ± 0.077	0.989 ± 0.010	0.0557 ± 0.0266	
Bone.......	0.184 ± 0.050	0.0549 ± 0.0079	0.0404 ± 0.0039	
Brain.......	0.140 ± 0.032	0.0111¶ ± 0.0028	0.00762 ± 0.00551	
Colon......	0.253 ± 0.065	0.0777 ± 0.0197	0.0430 ± 0.0123	
Heart......	0.187 ± 0.045	0.0240 ± 0.0040	0.00937 ± 0.00179	
Liver.......	0.242 ± 0.019	0.095 ± 0.020	0.0780 ± 0.0165	
Lung.......	0.256 ± 0.065	0.0657 ± 0.0144	0.0350 ± 0.0087	
Muscle.....	0.174 ± 0.047	0.0120 ± 0.0026	0.00910 ± 0.00645	
Pituitary...		0.0612 ± 0.0113	0.0128 ± 0.0035	
Skin........	0.230 ± 0.128	0.0410 ± 0.0071	0.0545 ± 0.0048	
Small bowel....	0.222 ± 0.056	0.0494 ± 0.0057	0.0355 ± 0.0142	
Spleen......	0.213 ± 0.074	0.0411 ± 0.0067	0.0337 ± 0.0013	
Stomach....	0.158 ± 0.037	0.0483 ± 0.0174	0.0312 ± 0.0055	
Testis......	0.201 ± 0.080	0.0174 ± 0.0034	0.0107 ± 0.0006	

* Seven animals used.
† Eleven animals used.
‡ Twelve animals used.
§ Nine animals used.
‖ Eight animals used.
¶ Two animals used.

Fig. 3 shows the data from the thyroid gland expressed as the ratio of the concentration of radioactivity in the gland to that of the average concentration in the body. This indicates the degree to which the sulfur injected as thiourea was concentrated in the thyroid gland. The standard error for each point is indicated on Fig. 3. These values were calculated by determining the ratios for each animal and then averaging them, not by comparing the average thyroid value and the average carcass value. This resulted in a smaller error. It will be noted that, although the maximum concentration in the thyroid was at 6 hours, the maximum relative concentration was at 48 hours.

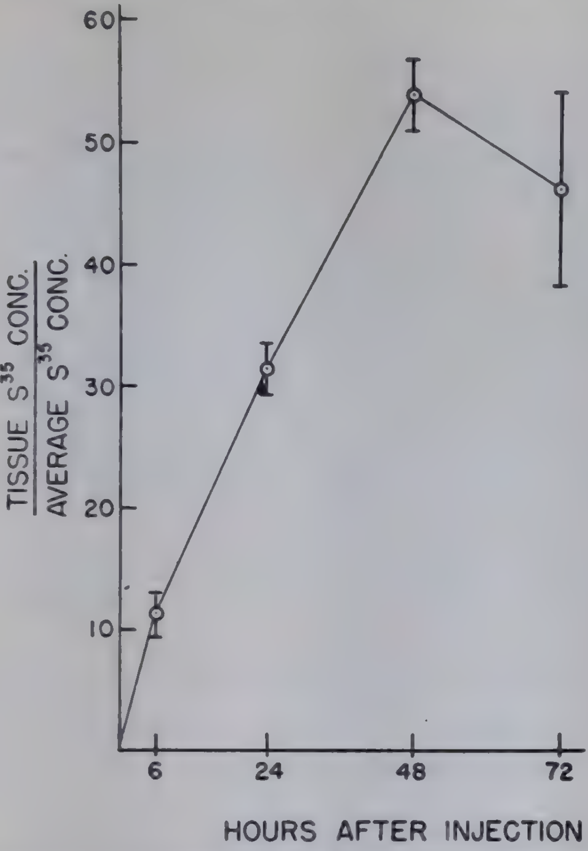

FIG. 3. Concentration of radioactivity in thyroid gland compared to average concentration of radioactivity in the body following injection of labeled thiourea.

TABLE II

Concentration of Radioactivity in Rats Injected with Radioactive Barium Sulfide and Sulfuric Acid

Injected material	Tissue	Tissue concentration at 24 hrs., per cent dose per gm.
Barium sulfide	Thyroid	0.121 ± 0.014
" "	Carcass	0.156 ± 0.021
Sulfuric acid	Thyroid	0.213 ± 0.057
" "	Carcass	0.236 ± 0.050

Table II shows the distribution of radiosulfur when it was injected as BaS and H_2SO_4. In neither case was the concentration higher in the thyroid gland than the average in the body.

DISCUSSION

After the intraperitoneal injection of thiourea labeled with radiosulfur, significant concentrations of radioactivity were found in the kidney and thyroid gland. The concentration in the kidney can be explained by the finding that the kidney was the principal route of excretion of the drug.

The concentration in the thyroid gland is best seen when compared with the average concentration in the body. This relative concentration reached a maximum 48 hours after injection. This was later than the peak of the absolute concentration, which was reached 6 hours after injection, indicating that the sulfur was held in the thyroid gland at a time when the concentration in the body as a whole was falling.

The chemical nature of the sulfur which was concentrated in the thyroid gland cannot be determined from these data. Whether it was thiourea or some metabolic product of thiourea is not known. Possible metabolic products are the sulfide or sulfate ions. Neither of these ions was concentrated when injected as such.

It has been shown that the injected thiourea was rapidly excreted in the urine, most of it unchanged. However, the excretion of about 10 per cent of the injected sulfur as sulfate indicates partial metabolism of the compound.

The data in this paper differ slightly from those given in a previous report (13). With material of specific activity sufficiently high to give significant counting rates even in very small organs, it was found that the concentration of activity in the pituitary gland was no higher than the average in the body.

The mechanism of the goitrogenic action of thiourea is still unknown, although several hypotheses have been advanced (14–17). A valid hypothesis must take into account the concentration of sulfur in the gland following the injection of thiourea. The partial metabolism of the compound in the body may also be of importance in explaining this action.

SUMMARY

Thiourea labeled with radioactive sulfur was injected into rats.

98 per cent of the activity was excreted in the urine within 48 hours.

Of the radioactivity in the urine, 6 per cent was in the form of inorganic sulfate and 6 per cent was in the form of ethereal sulfate. The major portion of the thiourea was excreted unchanged.

There was a 55-fold concentration of radioactivity in the thyroid gland.

We are indebted to Miss Frances Sharp for technical assistance, to Dr. D. L. Tabern of the Abbott Laboratories, and Dr. A. R. Ronzio and C. Wayne Bills of this laboratory for the preparation of the thiourea used in these experiments.

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STUDIES ON HYDROCARBONS STRUCTURALLY RELATED TO PHTHIOCEROL

SYNTHESIS OF THE LEVOROTATORY ENANTIOMORPH OF 4-METHYLTRITRIACONTANE

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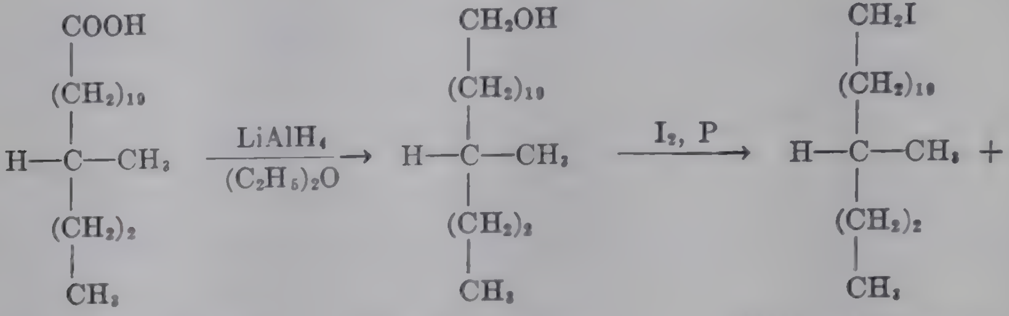
It has been shown previously (1, 2) that phthiocerane, the hydrocarbon derived from the wax alcohol phthiocerol present in the human and bovine types of tubercle bacilli (3–7), is a 4-methyl-substituted long chain hydrocarbon. The properties of phthiocerane were closely similar to those of synthetic DL-4-methyltritriacontane (1), but the former melted 0.5° above the melting point of the synthetic hydrocarbon, and there were slight differences in the appearance of the x-ray diffraction patterns. It was suggested that these differences might be caused by the presence of a small amount of a higher homologue (4-methylpentatriacontane) in the hydrocarbon derived from the natural product. Another suggested possibility was that phthiocerane, in spite of the absence of a detectable optical rotation (3), might be one of the enantiomorphs of 4-methyltritriacontane. It seems likely that only one of the two possible configurations of the asymmetric carbon atom carrying the methyl group is present in the natural product. If the reduction of phthiocerol gives a racemic hydrocarbon, this would suggest that one of the functional groups (hydroxyl or methoxyl) of the alcohol is attached at, or situated in the α position to, the carbon atom carrying the methyl side chain. It was thus of interest to compare the properties of phthiocerane with those of one of the enantiomorphs of 4-methyltritriacontane, and the synthesis of the levorotatory form has been undertaken, with D(−)-21-methyltetracosanoic acid (I), m.p. 60.6–60.8°, $[M]_D^{21} = -3.2°$, as initial material. The synthesis of I is described in another communication (8). The optically active acid (I) was converted into D(−)-*21-methyltetracosanol-1* (II) by reduction with lithium aluminum hydride in ether solution according to the method of Nystrom and Brown (9). The further steps in the synthesis are analogous to those used in the synthesis of the racemic hydrocarbons (1), as shown in the accompanying flow sheet.

The molecular rotations of the alcohol (II), the corresponding iodide (III), D(−)-*8-keto-30-methyltritriacontane* (V), and the levorotatory enantiomorph of 4-methyltritriacontane (VI) are all of the order of −3° to −4°; *i.e.*, of the same order of magnitude as that of I. The observed rotations are very small, however, and the molecular rotations cannot be given with great accuracy. In particular, the rotation for a 1 dm. tube of a 4.8 per cent solution of (−)-4-methyltritriacontane in chloroform is $\alpha_D^{22} = -0.03°$, showing that if phthiocerane is optically active its activity might have easily escaped detection.

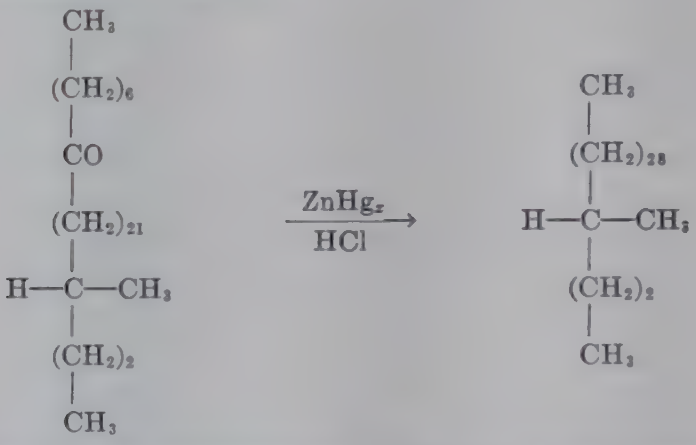


$$\begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{(CH_2)_{19}}\\ |\\ \mathrm{H{-}C{-}CH_3}\\ |\\ \mathrm{(CH_2)_2}\\ |\\ \mathrm{CH_3}\end{array} \xrightarrow[\mathrm{(C_2H_5)_2O}]{\mathrm{LiAlH_4}} \begin{array}{c}\mathrm{CH_2OH}\\ |\\ \mathrm{(CH_2)_{19}}\\ |\\ \mathrm{H{-}C{-}CH_3}\\ |\\ \mathrm{(CH_2)_2}\\ |\\ \mathrm{CH_3}\end{array} \xrightarrow{\mathrm{I_2,\ P}} \begin{array}{c}\mathrm{CH_2I}\\ |\\ \mathrm{(CH_2)_{19}}\\ |\\ \mathrm{H{-}C{-}CH_3}\\ |\\ \mathrm{(CH_2)_2}\\ |\\ \mathrm{CH_3}\end{array} +$$

(I) D(−)-21-Methyltetracosanoic acid (II) D(−)-21-Methyltetracosanol-1 (III) (−)-1-Iodo-21-methyltetracosane

$$\mathrm{CH_3(CH_2)_6COCH_2COOCH_3} \xrightarrow[\mathrm{CH_3CO(CH_2)_2CH_3}]{\mathrm{K_2CO_3}} \xrightarrow[\mathrm{CH_3OH}]{\mathrm{KOH,\ H_2O}}$$

(IV) Methyl 3-ketodecanoate



$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{(CH_2)_6}\\ |\\ \mathrm{CO}\\ |\\ \mathrm{(CH_2)_{21}}\\ |\\ \mathrm{H{-}C{-}CH_3}\\ |\\ \mathrm{(CH_2)_2}\\ |\\ \mathrm{CH_3}\end{array} \xrightarrow[\mathrm{HCl}]{\mathrm{ZnHg_x}} \begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{(CH_2)_{28}}\\ |\\ \mathrm{H{-}C{-}CH_3}\\ |\\ \mathrm{(CH_2)_2}\\ |\\ \mathrm{CH_3}\end{array}$$

(V) D(−)-8-Keto-30-methyltritriacontane (VI) (−)-4-Methyltritriacontane

The formulas are written according to Fischer's convention. The relative configurations of the iodide (III) and the hydrocarbon (VI) follow from the alcohol (II) and the ketone (V) respectively, but, as Fischer's convention does not apply to iodides and hydrocarbons, the prefix has been omitted for III and VI.

The thermal and x-ray data for (−)-4-methyltritriacontane are given in Tables I and II, the corresponding data for phthiocerane and DL-4-methyltritriacontane being included for comparison. The latter two were previously (1) found to possess two crystalline modifications, of which the one stable below 46° is identical with the orthorhombic (A) form of the normal chain hydrocarbons. The high temperature form, which is the stable form between a temperature of 46° and the melting point, does not, however, correspond to the hexagonal high temperature form of the *n*-hydrocarbons, the crystal structure instead being one of lower symmetry (with tilted chains). The same crystalline forms are found for (−)-4-methyltritriacontane, but the latter can also exist in a third form in which the chains are still more tilted towards the basal plane than in the high temperature form of the DL-hydrocarbon. For convenience, the different crystal modifications are called A, B, and C respectively, in order of increasing tilt of the chains. On crystallization from acetone the optically active hydrocarbon is obtained in the A form. The appearance of the specimens and the diffraction patterns are identical with those of the A form of the DL-hydrocarbon. Only three 00*l* reflections are observed. The A form

TABLE I

Thermal Data for Hydrocarbons

Compound	Crystals from	Transition point		M.p.	Solidification point	Remelting point
		On heating	On cooling			
		°C.	°C.	°C.	°C.	°C.
Phthiocerane	Acetone	45.6–46.8	46	59.1–59.3	59.0	59.1–59.3
DL-4-Methyltritriacontane	" Chloroform	45.6–46.4	46	58.6–58.7	58.5	58.6–58.7
(−)-4-Methyltritriacontane	Acetone	Below 46	46*	60.5–60.7†	58.4	58.5–58.6
	Chloroform		46*	61.6–61.8	58.4	58.5–58.6

* After solidification of the melt.

† The melting points of specimens crystallized from acetone vary somewhat, probably owing to the simultaneous formation of varying small amounts of the B form in the transition from the A form into the C modification on heating.

TABLE II

Crystal Data for Hydrocarbons

Compound	Crystal spacings					
	A form		B form		C form	
	Long spacing	Side spacings	Long spacing	Side spacings	Long spacing	Side spacings
	A	A	A	A	A	A
Phthiocerane	46.3	4.11, 3.70	42.4	4.36, 4.03		
DL-4-Methyltritriacontane	46.3	4.11, 3.70	42.2	4.36, 3.97		
(−)-4-Methyltritriacontane	46.2	4.12, 3.72	42.0	4.34, 4.03*	37.6	4.12, 3.86, 3.64

* At 55°.

may exist up to a temperature of 46°, at which point (or usually a few degrees lower) a transition occurs, mainly to the C form. Transition to the C form can also be induced at room temperature by mechanical treatment, *i.e.* pressing, of the specimen. The C form, which is stable up to its melting point at 61.6–61.8°, can be obtained from a slowly evaporating chloroform solution in the form of small lustrous plates. Under the microscope the latter are seen to be well developed single crystals, and pressed specimens give very good x-ray diffraction patterns showing many 00*l* reflections. The molten active hydrocarbon solidifies at 58.4° and remelting takes place at 58.5–58.6°, the form obtained on solidification evidently being identical with the high temperature form (B) of the DL-hydrocarbon. On cooling, the B form of the active hydrocarbon is transformed at about 46° to a mixture of the A and C modifications. On reheating a specimen from room temperature, melting occurred at 59–60°.

The DL-hydrocarbon on crystallization from chloroform is obtained in the A form. While the solvent rapidly evaporates from a chloroform solution of the active hydrocarbon, solutions containing the racemic hydrocarbon become covered with a thin transparent film which effectively retards further evaporation. Specimens of the DL-hydrocarbon obtained from acetone (A form) were previously reported (1) to give only three 00*l* reflections, but the films obtained on chloroform solutions give some weak but distinct higher order reflections, indicating a higher degree of regularity in the crystallographic direction within the films than in the pressed specimens.

The behavior of several mixtures containing the active and the DL-hydrocarbon in different proportions has been investigated. A mixture containing 15.2 per cent of the DL-hydrocarbon crystallized from chloroform in the C modification and melted at 59.9–60.2°. The solidification point was 58.3° and remelting occurred at 58.5°. A specimen containing 26.8 per cent of the DL-hydrocarbon crystallized under similar conditions was found to consist of a mixture of the A and C modifications. The melting point was 58.6–61°, and the solidification and remelting points were exactly the same as for the mixture containing 15.2 per cent of the DL-hydrocarbon. Finally, mixtures containing 52.8 and 67.1 per cent respectively of the DL-hydrocarbon were obtained from chloroform in the A modification and their thermal behavior was identical with that of DL-4-methyltritriacontane.

The results now described show that the high temperature (B) form of the racemic hydrocarbon is a racemic solid solution. They furthermore indicate that phthiocerane is a racemic or partially racemic hydrocarbon and that the difference in melting point between DL-4-methyltritriacontane and phthiocerane must be due to the presence of a small amount of a higher homologue in the latter. Finally, the results suggest that phthiocerol has at least one of the functional groups at, or in the α position to, the carbon atom carrying the methyl side chain.

EXPERIMENTAL[1]

D(−)-*21-Methyltetracosanol-1 (II)*—The reduction was carried out in the manner described by Nystrom and Brown (9). D(−)-21-Methyltetracosanoic acid (8) (0.96 gm.) was dissolved in 50 ml. of dry ether. 15 ml. of a 10 per cent solution of lithium aluminum hydride (Metal Hydrides, Inc.) in ether was added and the mixture refluxed for 1 hour. Water was added dropwise until the evolution of gas ceased. Dilute sulfuric acid was then added until the white precipitate had dissolved. The mixture was transferred to a separatory funnel. The ether layer was washed twice with water and dried by means of sodium sulfate. The ether was removed by distillation, leaving a beautifully crystalline residue. Crystallization from light petroleum gave 0.76 gm. (82 per cent of the theoretical) of D(−)-*21-methyltetracosanol-1* in the form of lustrous plates, m.p. 59.6–59.8°. Previously melted material showed the same melting point; long x-ray spacing (pressed specimen) 32.4 A. A melted specimen gave two sets of 00*l* reflections, corresponding to long spacings of 32.4 and 44.7 A respectively.

Optical Rotation—$\alpha_D^{22} = -0.07°$ (chloroform; *l*, 2 dm.; *c*, 3.8%); $[\alpha]_D^{22} = -0.9°$; $[M]_D^{22} = -3.2°$

Analysis—$C_{24}H_{50}O$ (354.6). Calculated. C 81.44, H 14.22
Found. " 81.21, " 14.26

(−)-*1-Iodo-21-methyltetracosane (III)*—D(−)-21-Methyltetracosanol-1 (0.70 gm.), iodine (0.28 gm.), and red phosphorus (0.0198 gm.) were kept at a temperature of 145° on an oil bath for 4 hours. The reaction product was extracted with ether and the ether solution washed with 5 per cent sodium hydroxide solution, followed by water. After the solution had been dried with sodium sulfate, the solvent was removed by distillation and the residue crystallized from 99.5 per cent ethanol, giving 0.81 gm. (88 per cent of the theoretical) of white microcrystalline (−)-*1-iodo-21-methyltetracosane.* The melting point was 43.2–43.5°, unchanged after previous melting; long x-ray spacing (pressed or melted specimen) 36.4 A.

Optical Rotation—$\alpha_D^{21} = -0.06°$ (chloroform; *l*, 2 dm.; *c*, 4.2%); $[\alpha]_D^{21} = -0.7°$; $[M]_D^{21} = -3.3°$

Analysis—$C_{24}H_{49}I$ (464.6). Calculated, I 26.52; found, I 26.59

D(−)-*8-Keto-30-methyltritriacontane (V)*—The active ketone was synthesized in the manner previously described for the racemic 4-methyl-substituted isomer (1), with 0.76 gm. of (−)-1-iodo-21-methyltetracosane, 0.33 gm. of methyl 3-ketodecanoate (10), 2.5 ml. of methyl-*n*-propyl ketone, and 0.8 gm. of potassium carbonate. The mixture was boiled under a reflux for 22 hours. The alkylation product was isolated and subjected to hydrolysis and ketonic cleavage by dissolving in a solution of potassium hydroxide (3 gm.), water (3 ml.), and methanol (45 ml.). The mixture was kept at a temperature of 50° for 30 hours. Part of the ketone formed separated in the hydrolysis mixture in the form of small lustrous plates. These were filtered off and found to weigh 0.18 gm. The filtrate was worked up in the ordinary manner, and crystallization from acetone gave 0.25 gm. of ketone. Total yield, 0.43 gm. or 52 per cent of the theoretical. The specimen of D(−)*-8-keto-30-methyltritriacontane* recovered from the hydrolysis mixture melted at 65.0–65.2°, solidified at 56.9°, and remelted at 57.1°. The low melting form is stable only near the melting point; melted material cooled to room temperature was found to possess the higher melting point. The material crystallized from acetone melted at 64.8–65.2°; long x-ray spacing (pressed specimen) 37.6 A, calculated from a very good photograph. A specimen prepared by means of the hot wire technique gave a long spacing of 46 A (vertical form), but the diffraction pattern also showed some faint lines corresponding to the 37.6 A spacing.

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[1] The thermal and x-ray investigations were carried out as described previously (1).

Optical Rotation—α_{D}^{23} = −0.04° (chloroform; *l*, 1 dm.; *c*, 5.5%); $[\alpha]_{\mathrm{D}}^{23}$ = −0.7°; $[\mathrm{M}]_{\mathrm{D}}^{23}$ = −3.6° ∼ −4°

Analysis—$C_{34}H_{68}O$ (492.9). Calculated. C 82.85, H 13.91
Found. " 82.83, " 13.87

(−)*-4-Methyltritriacontane* (*VI*)—The ketone just described (0.32 gm.) was reduced by Clemmensen's method (*cf.* (1)). 6 gm. of amalgamated zinc, 6 ml. of concentrated hydrochloric acid, and 2 ml. of glacial acetic acid were used initially. The zinc, hydrochloric acid, and glacial acetic acid were replaced by fresh reagents at 8 hour intervals. After a total of 80 hours the hydrocarbon was isolated and crystallized from acetone, giving 0.25 gm. of white microcrystalline material melting at 58–60° and solidifying at 57.6°. One further crystallization from acetone gave 0.22 gm. melting at 60–60.4°. The material solidified at 58.2° and remelted at 58.5°. 0.13 gm. of this material was treated with concentrated sulfuric acid at 120° for a period of 30 minutes. The recovered hydrocarbon melted, after crystallization from acetone, at 60.5–60.7°, solidified at 58.4°, and remelted at 58.5–58.6°. A detailed description of the thermal and crystal behavior of this material has already been given in the introduction.

Optical Rotation—α_{D}^{23} = −0.03° (chloroform; *l*, 1 dm. (micro tube); *c*, 4.8%); $[\alpha]_{\mathrm{D}}^{23}$ = −0.6°; $[\mathrm{M}]_{\mathrm{D}}^{23}$ = −3°

Analysis—$C_{34}H_{70}$ (478.9). Calculated. C 85.27, H 14.73
Found. " 85.10, " 14.68

We are indebted to Mr. W. Kirsten and Mr. I Alperowicz for the microanalyses, and to the National Swedish Antituberculosis Association and the Rockefeller Foundation for financial support.

SUMMARY

The levorotatory enanthiomorph of 4-methyltritriacontane has been synthesized and its thermal behavior and crystal structure compared with those of DL-4-methyltritriacontane and phthiocerane. The optically active hydrocarbon has three different crystalline modifications, two of which are identical with the high and low temperature forms respectively of the DL-hydrocarbon and of phthiocerane. It is shown that the high temperature form of DL-4-methyltritriacontane is a racemic solid solution and that phthiocerane is a racemic, or partially racemic, hydrocarbon. The results indicate that the difference in melting point between the DL-hydrocarbon and phthiocerane is due to the presence of a small amount of a higher homologue in the latter, and that phthiocerol has at least one of the functional groups situated at, or in α position to, the carbon atom carrying the methyl side chain.

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METABOLISM OF TESTOSTERONE BY LIVERS OF DIFFERENT SPECIES OF ANIMALS*

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In previous papers (1–4) it has been demonstrated that the metabolism of testosterone by liver tissue involves at least two enzyme systems, one requiring diphosphopyridine nucleotide (DPN) and the other citrate as cofactor. The former compound is involved in the oxidation of the alcohol group on C-17 to a ketone; the latter increases the rate of destruction of the α,β conjugation in Ring A formed by the Δ_4 double bond and the ketone group on C-3. The variation in testosterone metabolism has been investigated in a wider series of species, and evidence of a phylogenetic development of the enzyme systems has been found.

Methods

Except in the case of the steer and the human, liver tissue was removed in the laboratory immediately after death by decapitation. Human liver tissue was removed during surgery and immediately immersed in ice-cold Locke's solution. It was then transported to the laboratory as rapidly as possible. Steer liver was obtained at the slaughter-house, cooled, and also brought to the laboratory within a short time. The tissue was immediately sliced with a razor or minced with a mechanism made up of multiple razor blades. It was then suspended in the buffer solution of testosterone and incubated in an atmosphere of oxygen, as previously described. Enzymic action was stopped by boiling the contents of the flask under a reflux for 20 minutes, after which extraction and analysis were carried out as in previous studies (5).

60 per cent DPN was procured from the Schwarz Laboratories. It was added directly to the flasks to give a final concentration of 0.001 M DPN. Sodium citrate was used at a concentration of 0.001 M.

The concentration of molecules containing the α,β-unsaturated ketone group in Ring A was estimated by the ultraviolet absorpton band at 240

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TABLE I

Destruction of Testosterone by Livers of Different Species

			Buffer only		Buffer + DPN		Buffer + citrate		Buffer + DPN + citrate	
Animal	No. of livers combined	Original testosterone concentration	α,β destruction	17-Ketosteroids formed	α,β destruction	17-Ketosteroids formed	α,β destruction	17-Ketosteroids formed	α,β destruction	17-Ketosteroids formed
		γ per flask	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*	γ per gm. per hr.*
Fish	6	200	64 (46– 92)	0	56 (30– 70)	0	62 (38– 76)	0		
Frog	5	200	61 (54– 69)	0						
Turtle	5	200	31 (16– 42)	0	32 (22– 38)	0	41 (14– 62)	0		
Rattlesnake	2	200	39 (26– 62)	0	66 (43– 89)	0	40 (37– 81)	0		
Chicken	1	576	380 (350–406)	190 (163–241)	520 (485– 588)	337 (312–359)	634 (580–666)	135 (107–167)	980 (930– 1015)	261 (236–292)
Steer	1	200	7? (0–22?)	0	10? (0– 29)	251 (208–306)	74 (50– 96)	0	124 (106– 143)	151 (144–158)
Rat fed†	3	200	98 (96–100)	17 (11– 23)	234 (211– 266)	62 (62– 62)	184 (166–197)	22 (13– 27)	>273 (>252–>294)	45 (40– 50)
" fasted 48 hrs.†	3	200	47 (39– 54)	24 (18– 31)	153 (145– 162)	106 (106–107)	101 (88–108)	22 (14– 28)	215 (174– 240)	69 (42– 92)
" fed, ♀	4	288	202 (201–204)	16 (11– 20)	>367 (>365–>367)	91 (87– 97)	304 (303–304)	26 (21– 30)	>365 (>358–>367)	55 (17– 76)
" " ♂	5	288	195 (178–212)	72 (55– 88)	261 (248– 270)	181 (144–216)	316 (296–351)	42 (31– 53)	357 (352– 362)	112 (98–124)
Rabbit	2	288	181 (170–193)	96 (86–105)	158 (150– 167)	97 (93–100)	255 (250–259)	36 (33– 42)	264 (260– 266)	65 (63– 66)

Guinea pig	3	288	96 (52–149)	104 (98–111)	168 (150– 179)	134 (102–174)	155 (152–160)	27 (24– 33)	281 (256– 298)	52 (36– 65)
Dog, ♂	1	288	91 (78–104)	31 (28– 37)	154 (138– 164)	100 (97–104)	117 (104–127)	31 (28– 34)	246 (239– 254)	49 (44– 52)
Monkey	1	288	155 (146–162)	133 (122–148)	210 (205– 219)	170 (147–190)	284 (282–287)	122 (118–128)	295 (284– 306)	101 (90–112)
Human	6	200	137 (106–196)	52 (40– 68)	164	153				
"	1	288	224 (223–226)	132 (118–145)	183	270	291	95 (86–104)	312 (306– 317)	224 (206–242)
"	1	288	305 (294–316)	80 (66– 94)	466 (386– 547)	183 (180–186)				

Except in the case of the fish and turtle, in which data from three incubations are combined in each case, each line represents a single incubation. The number of livers in all cases except the two mentioned represents the numbers which were minced together to furnish sufficient sample for the series. The numbers in parentheses indicate the range of values obtained from individual incubation flasks. Each flask contained 25 ml. of buffer and 0.50 to 1.00 gm. of liver mince. In most cases the results from three flasks have been averaged to give the mean value. Testosterone concentration is given because, over the range involved, destruction varies almost directly as substrate concentration. This must be considered in comparing the rates of destruction.

* Micrograms of steroid per gm. of liver mince per hour.

† These rats were from the same litters.

mμ. The 17-ketone group was determined by the absolute alcohol method of Callow, Callow, and Emmens (6). Spectrophotometric readings were made at 440, 520, and 600 mμ wave-lengths. These were corrected for background absorption on the assumption that it showed a constant fall over this range. When the three wave-lengths mentioned were used, the brown color of testosterone in the quantities employed gave no significant value after correction.

Results

The results of studies of the various species are summarized in Table I. The livers of fish, amphibians, and reptiles all destroyed the α,β structure in testosterone at a slow rate, but no evidence of oxidation at C-17 was found. The addition of either DPN or citrate did not affect the result significantly. The enzyme system involved, therefore, apparently does not require these compounds as cofactors.

A typical series of experiments on one form of reptile, the turtle, is shown in Table II. While the activity of slices was approximately twice that of the minced tissue from any one individual, the reaction to the addition of cofactors was the same. There did not seem to be any acceleration in the presence of DPN or citrate. The variation between turtles of the same sex was as great as that between turtles of opposite sexes.

With birds and mammals, however, enzyme systems requiring each of these cofactors appear. If DPN was added to minces or slices of livers from members of these orders, 17-ketosteroids were formed in all animals except the rabbit. In the latter, 17-ketosteroids were produced in the presence of the liver tissue but the nucleotide seemed to have no effect on this process.

In addition to the effect on C-17, the presence of DPN increased the destruction of the conjugated system in Ring A as measured by the decrease in absorption at 240 mμ, again with the exception of the rabbit. This would appear to be due to the ability of an enzyme to reduce the ketonic group on C-3 more readily when there is a ketone rather than an alcohol on C-17. When the 17-ketosteroids formed by chicken liver, which has a relatively high concentration of this oxidative system, were treated with succinic anhydride, all were found in the water-soluble fraction. They must, therefore, have had an alcohol group as well as the 17-ketone. The most likely assumption is that the ketone on C-3 was reduced to an alcohol and formed Δ_4-androstenol-3-one-17.

As can be seen in Table I, in all species except the steer the number of conjugated groups disappearing equals or exceeds the number of ketonic groups formed at the other end of the molecule. This was true both before and after the addition of exogenous DPN. The problem of the rôle of the 17-ketosteroids has been analyzed in the case of chicken liver. This tissue forms relatively large amounts of alcoholic 17-ketosteroids from testosterone without added cofactor.

In Table III the effect of time on the proportions of the different fractions is demonstrated. The non-conjugated non-17-ketosteroids represent the difference between Columns 1 and 2. When incubation time was increased, the concentration of the 17-ketosteroids remained relatively constant but the amount in Column 3 increased. These observations could best be explained if the alcoholic 17-ketosteroids were themselves intermediates which underwent a reduction at C-17.

TABLE II

Metabolism of Testosterone by Turtle Liver

The change is given as micrograms per gm. per hour.

Temperature	No. and sex	Preparation	Buffer only*	Buffer + 0.001 M DPN	Buffer + 0.001 M citrate	Buffer + 0.001 M DPN + 0.001 M citrate
			α,β groups destroyed	α,β groups destroyed	α,β groups destroyed	α,β groups destroyed
°C.						
30	1 ♂	Mince	42	38	42	
30	1 ♂	Slices	61		66	
30	2 ♂	Mince	16		14	14
30	2 ♂	Slices	70		59	
38.5	3 ♂	Mince	21	30	29	
30	4 ♀	"	40	38	62	
30	4 ♀	Slices	95		85	
30	5 ♀	Mince	34		57	
Average.............		Mince	31	35	41	
"		Slices	75		70	

* The buffer solution contained KCl 0.0056 M, $MgCl_2$ 0.0021 M, NaCl 0.08 M, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.4) 0.04 M, glucose 0.1 per cent. Approximately 1 gm. of mince or slices was used per flask together with 200 γ of testosterone and 25 ml. of buffer solution. The extracts from all incubations were analyzed for 17-ketosteroids but none were found.

The sequential relationship of the formation of 17-ketosteroids and their breakdown was investigated by comparing the effect of chicken and rat livers. In the absence of added DPN rat livers form little 17-ketosteroids but break down many more α,β groups. Testosterone was first incubated with mince from one species of liver and the products were then incubated with liver mince of the other species. Analyses for α,β conjugation and 17-ketosteroids were made at the end of each incubation. The results are shown in Table IV. It is evident that bird liver was unable to form 17-ketosteroids from the products of the action of rat liver on testosterone. The small amounts present could be accounted for by conversion of the residual testosterone. When the order was reversed, however, rat liver was able to convert the 17-ketosteroids formed by the bird liver into non-ketonic unconjugated compounds. This would seem to confirm the previous evidence that the formation of 17-ketosteroids might be an intermediate step.

TABLE III

Effect of Incubation with Chicken Liver Mince on Different Parts of Testosterone Molecule

The tissue was incubated with 400 γ of testosterone in glucose buffer.

Flask No.	Amount of liver	Incubation time	α,β groups destroyed (1)	Alcoholic 17-ketosteroids formed (2)	Non-conjugated non-17-ketosteroids formed (3)
	gm.	*min.*	*per cent*	*per cent*	*per cent*
1	0.975	60	53	39	14
2	0.990	60	62	35	27
3	1.180	120	80	39	41
4	1.110	120	87	44	43

TABLE IV

Balance Sheet for Reincubation Experiment in Which Both Rat and Chicken Livers Were Used in Series

The 17-ketosteroids formed by chicken liver were destroyed by rat liver.

Incubation		Rat liver followed by chicken liver		Chicken liver followed by rat liver	
		γ	γ	γ	γ
1st	Testosterone added	150	150	150	150
	" recovered	64	60	10	13
	" destroyed	86	90	140	137
	17-Ketosteroids formed	0	0	107	104
2nd	Testosterone present, start	64	60	10	13
	17-Ketosteroids present, start	0	0	107	104
	Testosterone recovered	37	38	0	0
	17-Ketosteroids recovered	24	23	40	40
	Testosterone destroyed	27	22	10	13
	17-Ketosteroids destroyed			67	64
	" formed	24	23		

When citrate was added to any of the tissue preparations of birds or mammals, there was a significant, often a marked, increase in the destruction of the α,β structure. As shown previously (3), this appears to be a specific effect of citrate. There was no effect in the other orders of animals.

In addition, there was usually a definite decrease in 17-ketosteroid formation if these would otherwise have been formed. One exception was the monkey, in which there was little effect on the 17-ketosteroids but a marked increase in α,β bonds destroyed. This second effect of citrate appears to be unrelated to the first, as can be seen in Table I.

A comparison of the results when both citrate and DPN were added with those when either was used alone indicates that the effects were roughly the algebraic sum of the individual influences. The destruction of α,β groups was greater than with either alone, while the concentration of 17-ketosteroids was intermediate. Within mammalian species themselves there was considerable variation in the activity of the different enzyme systems.

The liver from only one herbivore was investigated, that of the steer. In Table I it will be seen that there was little or no destruction of the conjugated system or oxidation at C-17 by the mince alone. The addition of DPN led to a marked formation of 17-ketosteroids but no significant change in Ring A. Addition of citrate brought about the change in α,β structure but no oxidation at the other end of the molecule. If both cofactors were added, changes occurred in both positions. The relative inactivity of steer liver minces and extracts, together with the apparently single action in the presence of each cofactor alone, has been repeatedly observed. This would seem to indicate either a low level of these cofactors in the original tissue or a very rapid destruction during preparation. Apparently the enzyme system which does not require either of these cofactors and which is present in the livers of the lower orders is not found in steer liver, or else some as yet unknown cofactor is absent.

Rabbit liver seems to show a more fundamental difference from the liver of other mammals. While the unsupplemented liver both destroyed the α,β structure and formed 17-ketosteroids, the addition of DPN had no influence on the rate of either reaction. Citrate increased the destruction of α,β groups and depressed the formation of 17-ketosteroids, but the simultaneous addition of DPN had no significant additional effect. These observations would all be true if triphosphopyridine nucleotide rather than DPN were the cofactor in this system. The definitive experiment with added triphosphopyridine nucleotide has not yet been done.

DISCUSSION

The evidence here presented would indicate that, associated with the development of homeothermic control, there was an increase in the complexity of the enzyme systems degrading the gonadal steroids.

In the poikilotherms body temperature is ordinarily lower than that of the homeotherms and chemical reactions, both synthetic and degradative, would be slower in the presence of the same concentrations of substrate and catalyst. The turn-over of such active compounds as the sex steroids would therefore be less, and the need for complex and rapid adjustment rare. Moreover, the changes in temperature of the organism due to season and environment themselves regulate the production of hormones. A simple system of inactivation would be sufficient.

In the homeotherms, however, production of sex steroids is both more rapid and more complex. Cyclic phenomena occur under conditions of a constant internal physical environment. In turn more rapid inactivation mechanisms have been developed. These, however, require labile energy transfer systems. Thus, while destruction may be more rapid, it also becomes subject to the supply of these systems. A more rapid, more adaptable, but also more labile system has thus evolved.

SUMMARY

The ability of liver minces and slices from various species and orders of animals to destroy testosterone has been investigated, both in the absence and in the presence of added diphosphopyridine nucleotide and citrate.

In fish, amphibians, and reptiles there appears to be an enzyme system which slowly destroys the conjugated double bond system in Ring A but does not form 17-ketosteroids. No system, the activity of which was increased by DPN or citrate, was identified.

In birds and mammals, systems requiring DPN and citrate are present. The DPN-catalyzed reaction leads to the formation of 17-ketosteroids, while that requiring citrate acts on the α,β conjugation in Ring A without forming 17-ketosteroids. The formation of 17-ketosteroids in the presence of DPN is also decreased by citrate.

The relative concentrations of the two systems requiring cofactors, as well as cofactor availability, vary between different species. The chicken appears to have a relatively high concentration of the DPN-activated system, man and the guinea pig an intermediate balance, and the rat and dog a relatively high activity of the citrate-activated enzyme. Steer liver shows little activity unless cofactors are added, but destruction occurs in the presence of either of the two compounds studied which play this rôle. This would indicate a relative unavailability of these substances, at least under the conditions of preparation of the tissue. Rabbit tissue seems to use some cofactor other than DPN in the oxidation of C-17.

The relation of the development of the systems activated by DPN

and citrate to the appearance of homeothermic mechanisms is discussed. A tentative outline of the possible order of enzymic reactions is also given.

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THE SEDIMENTATION BEHAVIOR OF BOVINE AND EQUINE IMMUNE PROTEINS*

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In previous studies, we have described the isolation, by chemical methods, and some chemical and physical properties of immune proteins from the cow and the horse (1–4). In extension of this work, the sedimentation behavior of these same preparations has been studied in the ultracentrifuge. It was found that the principal component of these proteins has a sedimentation constant of about 7 Svedberg units[1] and in association with this there regularly occurs another component of about 10 Svedberg units. While most of our observations are in good accord with those of previous investigators, some minor differences have been found and these will be discussed following the presentation of our data.

Methods and Materials

The sedimentation studies were made in the Spinco[2] electrically driven ultracentrifuge. A brief description of this instrument, and the controls and procedures as used in this laboratory, has been given in a previous publication (5). All of the runs were made at 59,780 R.P.M., equivalent to centrifugal fields of 240,000 × *g* and 300,000 × *g* at the meniscus and base.

The isolation of the bovine proteins of plasma, colostrum, and milk has been described (1, 2). Preparation A of bovine γ-globulin was derived from normal steer blood obtained at the slaughter-house. Preparation B was isolated from the plasma of cows hyperimmunized with a mixture of antigens: diphtheria toxin (or toxoid), vaccinia virus, and a killed culture of *Hemophilus pertussis;* the colostrum, milk, and T-globulins were from the same animals (6).

The immune globulins of the horse used in this study are those previously isolated by Smith and Gerlough (4), and contained high titers of tetanus

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[1] The Svedberg unit is defined as 1×10^{-13} sq. cm. per second, and is abbreviated as S.

[2] Specialized Instruments Corporation, Belmont, California.

antitoxic activity. All of the preparations used in this study had been dried from the frozen state and were studied after standing in dry form for 1 to 3 years. Samples which were salt-free were dissolved in the appropriate solvent immediately before use; others were dialyzed at 2° until they were in equilibrium with the solvent.

Results

Bovine Proteins—Measurements on these proteins are presented in Table I. The sedimentation constants were found to be essentially independent of the protein concentration within the limits studied. It is apparent that none of these proteins is molecularly monodisperse, and that they are all very similar in composition. In each instance, the main component possesses a sedimentation constant ($s_{20,\,w}$) near 7 Svedberg units and represents 80 to 90 per cent of the total refractive increment. This is always accompanied by smaller amounts of a second component which has a value of about 10 S; this second particle size represents about 10 to 20 per cent of the total sedimenting area. Two of our preparations also show the presence of additional substances. The colostrum pseudoglobulin contains about 10 per cent of slowly sedimenting material (about 2 S). The colostrum euglobulin contains about 5 per cent of heavy material which is detectable only at higher protein concentrations.

Hansen, Potter, and Phillips (7) reported that colostrum pseudoglobulin isolated by the method of Smith (1) showed only a single sedimenting boundary with a value of $s_{20,\,w} = 7$ S. This differed from the behavior of such material previously reported by one of us in preliminary observations (2), and from that observed in the present study. At least two components were always observed in our pseudoglobulin preparations obtained from either milk or colostrum. Dr. R. G. Hansen (now of the University of Utah) kindly gave us a dried sample of colostrum pseudoglobulin which was prepared by him at the University of Wisconsin by the same method which we used (1). On examination in the ultracentrifuge, there was no doubt of the presence of two components, although the second one was present in smaller amounts than in our preparations. A sedimentation diagram of Hansen's preparation is shown in Fig. 1, *A* in comparison with our colostrum and milk pseudoglobulins (Fig. 1, *B* and *C*). The milk and colostrum euglobulins are shown in Fig. 1, *D* and *E*.

Our preparations of the bovine plasma globulins (γ and T) likewise contain two components, in agreement with earlier observations (2). However, Hess and Deutsch (8) reported that they obtained from normal cows a γ_2-globulin (equivalent in electrophoretic mobility to our γ-globulin) which was essentially monodisperse ($s_{20,\,w} = 7.4$ S), while their γ_1-globulin (equivalent to our T-globulin) contained about 4 per cent of heavier con-

TABLE I

Sedimentation Behavior of Bovine Immune Proteins

The values of the sedimentation constants (S_1, S_2, S_3, and S_4) are for $s_{20,w}$ in Svedberg units and incorporate the usual corrections for the viscosity and density of the medium. The temperature is the average for the period during the run when photographs were being taken. The parenthetical values give the amount of each component as estimated from its percentage of the total sedimenting area. All of these runs were performed with 0.15 M sodium chloride as the solvent.

Protein	pH	Protein concentration	Temperature	S_1	S_2	S_3	S_4
		per cent	°C.				
Milk pseudoglobulin	6.7	1.1	25.2		6.8 (85%)	8.9 (15%)	
“ “	6.2	1.0	30.0		6.8 (87%)	9.5 (13%)	
Colostrum pseudoglobulin	6.8	0.25	23.7	2.0 (9%)	6.8 (77%)	10.0 (15%)	
“ “	5.8	0.5	25.5	1.4 (11%)	6.8 (77%)	10.0 (12%)	
“ “	5.8	1.0	23.9	2.1 (11%)	6.9 (78%)	10.5 (11%)	
“ “ *	6.6	1.0	23.6		7.4 (91%)	10.0 (9%)	
Milk euglobulin	6.2	1.2	23.7		6.8 (86%)	9.7 (14%)	
“ “	6.2	0.9	26.7		6.9 (82%)	10.8 (18%)	
Colostrum euglobulin	6.2	0.25	25.3		7.2 (78%)	10.2 (22%)	
“ “	6.1	0.5	23.4		7.2 (80%)	10.4 (20%)	
“ “	6.1	1.0	24.1		6.8 (77%)	9.6 (18%)	17.6 (5%)
Plasma T-globulin	6.0	0.25	24.8		6.9 (80%)	9.9 (20%)	
“ “	6.0	0.5	22.5		6.9 (89%)	9.7 (20%)	
“ “	6.2	1.0	23.1		6.9 (86%)	9.5 (14%)	
Plasma γ_1-globulin†	7.0	1.1	23.2		6.8 (91%)	9.2 (9%)	
Plasma γ-globulin A	6.7	0.25	18.7		6.9 (79%)	10.8 (21%)	
“ “	6.7	0.5	23.9		7.0 (81%)	9.5 (19%)	
“ “	6.7	1.0	22.7		7.0 (84%)	10.5 (16%)	
Plasma γ_2-globulin†	6.6	1.0	24.0		7.0 (85%)	10.2 (15%)	
Plasma γ-globulin B	6.2	0.25	24.8		6.9 (80%)	9.9 (20%)	
“ “	6.2	0.5	22.5		6.9 (89%)	9.7 (11%)	
“ “	6.2	1.0	23.1		6.9 (86%)	9.5 (14%)	

* This sample was prepared from a normal animal by Dr. R. G. Hansen.

† These samples were provided by Dr. H. F. Deutsch of the University of Wisconsin. In his nomenclature, these samples, identified as γ_1- and γ_2-globulins, are equivalent to our T- and γ-globulins respectively.

stituents with $s_{20,\,w} = 9$ S. Later, these investigators (9) obtained similar results with globulins prepared from hyperimmunized animals. Examination of samples kindly furnished to us by Dr. Deutsch indicates that the γ_1 preparation contains 9 per cent of the heavier component, and that the γ_2 preparation contains 16 per cent of the heavier component. Sedimentation patterns of the preparations made by Hess and Deutsch are shown in Fig. 1, *F* and *G* in comparison with ours (Fig. 1, *H* and *I*).

It must be noted that Hess and Deutsch studied their preparations immediately after fractionation, while our studies of their material were performed on dried preparations which had been kept by us for about a year or longer.

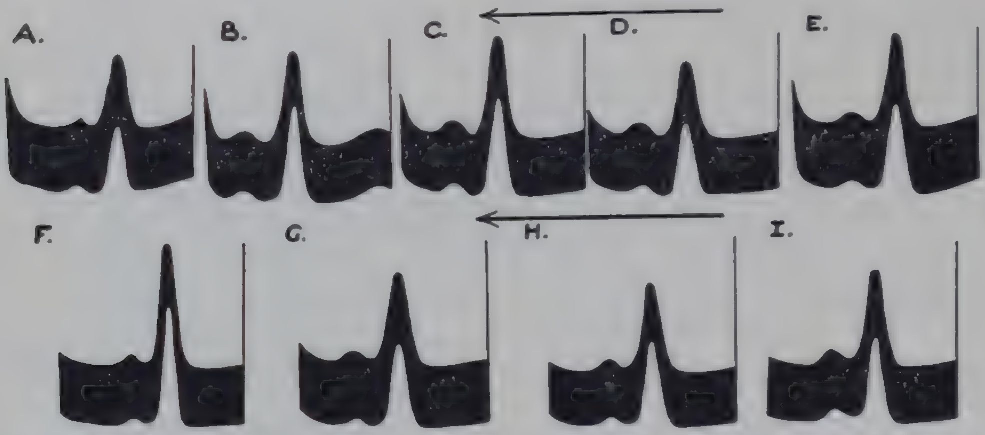

FIG. 1. Sedimentation patterns obtained on various bovine immune proteins *A* is a colostrum pseudoglobulin (Hansen) at 1.0 per cent; *B* is colostrum pseudoglobulin at 1.0 per cent; *C* is a milk pseudoglobulin at 1.1 per cent; *D* and *E* are milk (1.2 per cent) and colostrum (1.0 per cent) euglobulins (the colostrum euglobulin pattern does not show the heaviest component, $s = 17.6$ S); *F* and *G* are γ_1- and γ_2-globulins prepared by Hess and Deutsch (8); *H* and *I* are γ- and T-globulins at 1.0 per cent concentration.

All of the results discussed thus far were obtained on preparations isolated by chemical procedures. Recently, the separation of bovine serum proteins was accomplished by an electrophoresis-convection method (10). It was of considerable interest to examine the sedimentation behavior of some of these proteins. Dr. Cann, Dr. Brown, and Dr. Kirkwood very kindly permitted us to study two of their bovine γ-globulin preparations: Fraction E possessing an electrophoretic mobility of -1.24×10^{-5} sq. cm. per volt per second (in veronal buffer at pH 8.7) and Fraction G with a mobility of -2.25×10^{-5}. Their respective isoelectric points are 7.31 and 5.74; these preparations appear to correspond to γ-globulin (γ_2) and T-globulin (γ_1). Samples of these were furnished in lyophilized form and were dialyzed against 0.15 M sodium chloride.

Results on these preparations are given in Table II and representative diagrams are shown in Fig. 2. It is evident that the results on these fractions are similar to those obtained on the chemically isolated ones in that the same two components are present: a large one with an $s_{20,\,w}$ of about 7 S and a secondary one of about 10 S. However, it should be noted that the main component of Fraction E shows a pronounced concentration effect leading to an extrapolated value at zero concentration of 7.3 S.

Equine Proteins—Table III gives the results obtained in the ultracentrifuge on the tetanus antitoxin preparations. The γ- and T-globulins show results very similar to those obtained with the bovine proteins in that two components were found and these possess similar sedimentation constants. The equine γ-globulin, like most of the bovine globulins, shows little or no effect of protein concentration on the sedimentation constants of the various components. In contrast to these, the equine T-globulin does show a marked concentration effect. The sedimentation constants found on extrapolation to zero concentration are 7.3 S for the main component and 10.3 S for the secondary component. This suggests that the components of the T-globulin may be much more asymmetrical than those of the γ-globulin.

TABLE II

Sedimentation of Bovine γ-Globulins Prepared by Electrophoresis-Convection

The sedimentation constants are given in Svedberg units. The solvent was 0.15 M sodium chloride. Fraction G corresponds to γ_1- or T-globulin and Fraction E to γ- or γ_2-globulin.

Fraction	pH	Protein concentration	Temperature	S_1	S_2
		per cent	°C.		
G	8.5	0.25	28.9	6.7 (86%)	9.8 (14%)
	8.5	0.5	29.7	6.8 (84%)	10.6 (16%)
	8.5	1.0	27.3	6.7 (91%)	9.6 (9%)
E	8.0	0.25	28.8	7.1 (91%)	9.8 (9%)
	7.6	0.5	30.5	6.9 (94%)	10.5 (6%)
	8.0	0.8	29.2	6.7 (90%)	10.0 (10%)
	8.0	0.8	29.4	6.7 (90%)	10.0 (10%)

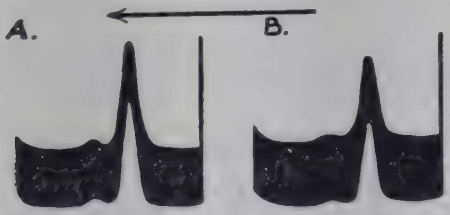

FIG. 2. Sedimentation patterns of bovine γ-globulins prepared by electrophoresis-convection (10). *A* is Fraction E at 0.8 per cent, and *B* is Fraction G at 1.0 per cent.

It was of considerable interest to examine Fraction IV-C2, since previous study (4) had indicated that this pseudoglobulin fraction was higher in tetanus antitoxic activity than the γ- and T-globulins obtained from the same plasma. Electrophoretically, Fraction IV-C2 contains no γ-globulin and only about 1 per cent T-globulin and is mainly a mixture of β- and α-globulins with peak mobilities between 3.0 and 4.9 × 10^{-5} sq. cm. per volt per second in veronal buffer at pH 8.5. In the ultracentrifuge,

TABLE III

Sedimentation Behavior of Equine Immune Proteins

The conditions were the same as those given in Table I. All of the measurements were made with 0.15 M sodium chloride except those marked with an asterisk, which were performed with 0.1 M veronal buffer. The sedimentation constants are in Svedberg units.

Protein	pH	Protein concentration	Temperature	S_1	S_2	S_3
		per cent	°*C.*			
T-Globulin	6.2	0.25	24.0		6.8 (78%)	9.9 (22%)
"	5.8	0.25	24.0		7.2 (77%)	10.2 (23%)
"	6.1	0.5	24.4		6.9 (81%)	10.3 (19%)
" *	8.5	0.9	22.3		6.7 (88%)	9.4 (12%)
"	5.5	1.0	22.4		6.6 (81%)	9.8 (19%)
γ-Globulin	5.8	0.25	24.4		6.6 (88%)	8.4 (12%)
"	5.8	0.5	24.4		6.7 (87%)	9.0 (13%)†
" *	8.5	0.7	21.7		6.5 (91%)	9.9 (9%)
"	5.0	1.0	26.5		6.5 (93%)	8.8 (7%)
"	5.2	1.0	26.6		6.8 (93%)	9.2 (7%)
Globulin‡	5.7	0.5	24.9	6.2 (31%)	7.5 (60%)	14.8 (9%)
"	5.6	0.5	28.4	5.6 (40%)	7.6 (53%)	15.0 (7%)
" *	8.5	0.9	24.3	4.8 (24%)	6.9 (68%)	11.4 (8%)
"	8.8	1.0	24.3	6.0 (27%)	7.1 (62%)	8.4 (11%)
"	5.3	1.2	26.1	5.3 (32%)	6.5 (59%)	7.9 (9%)

† This component showed some evidence of splitting into two.

‡ Fraction IV-C2.

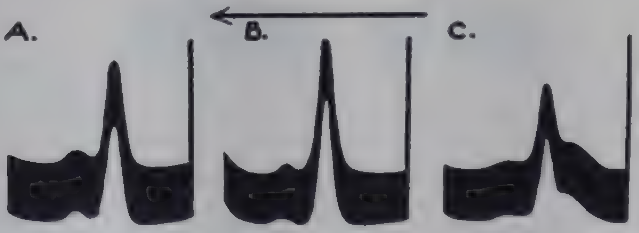

FIG. 3. Sedimentation behavior of equine immune fractions. *A* is a T-globulin at 1.0 per cent concentration, *B* is a γ-globulin at 1.0 per cent, and *C* is Fraction IV-C2 at 1.2 per cent.

Fraction IV-C2 contains three well defined components, the main one having a value of $s_{20,w}$ = 8.2 S when extrapolated to zero concentration. The heaviest component also shows a significant variation of $s_{20,w}$ with concentration; at zero concentration $s_{20,w}$ = 20 S. The lightest component shows considerable variability both in amount and sedimentation constant and shows some evidence of being a mixture. Representative sedimentation diagrams of γ- and T-globulins and Fraction IV-C2 are shown in Fig. 3.

It should be recalled that some investigators (11, 12) have observed the presence of components with $s_{20,w}$ = 20 S in certain antibody preparations obtained from cow and horse. Such heavy components have also been found in human γ-globulin preparations (13, 14). In this investigation, most of the preparations did not contain such heavy components and similar findings have also been reported earlier (2, 8, 9, 15). The only samples which do contain such components are euglobulin from colostrum and the equine Fraction IV-C2. However, there is no evidence as yet that antibody activity is associated with these heavy components present in our preparations.

Deutsch and Nichol (15) have also prepared γ-globulins from normal and hyperimmune horse sera by chemical fractionation. Their sedimentation studies gave results very similar to those reported here; 80 per cent of the globulin had a sedimentation constant of 6.8 to 7.2 S and about 20 per cent sedimented in the range of 8 to 15 S.

DISCUSSION

Some earlier studies have been made on the sedimentation behavior of immune fractions obtained by precipitation with specific antigens. Kabat (11, 12) found that antipneumococcus antibodies of the cow and horse are mainly heavy molecules with $s_{20,w}$ = about 20 S, and a molecular weight of about 1 million. In contrast, it has been found that undigested diphtheria antitoxin of the horse has a sedimentation constant near 7 S and a molecular weight of 160,000 to 180,000 (16–18). Thus, the main component of the γ and T preparations of tetanus antitoxin studied in this investigation is similar to the diphtheria antitoxin. The observations of Deutsch and Nichol (15) on globulins containing both tetanus and diphtheria antitoxins are in agreement with these studies. It is likely that the antitoxic component of our Fraction IV-C2 is also of about the same size.

Most of the bovine globulins studied in this investigation do not contain detectable amounts of heavy molecules with $s_{20,w}$ = about 20 S, although considerable hyperimmunization was produced to the three different kinds of antigens. It is important to reemphasize earlier statements that the immune proteins obtained from milk and colostrum or from plasma do not differ in size (2, 3).

The occurrence in all of our bovine and equine samples of a main component of about 7 S with smaller amounts of a component of about 10 S is quite striking and was described earlier in preliminary observations of the bovine proteins (2). It has been suggested that these components (and also the component of 20 S) may simply have a polymeric relationship to one another (2, 14). Thus the protein of 7 S has a molecular weight of about 180,000, and the protein of 10 S would be about double the size. While there has been some suspicion that the component of 10 S may have been produced by the chemical fractionation procedure or by prolonged standing after lyophilization, there is no direct evidence that this is the case. The dried bovine fractions of Cann, Brown, and Kirkwood (10) obtained by a mild physical separation are very similar in sedimentation pattern to those obtained by distinct chemical methods. While lyophilization by itself has not been excluded as leading to the formation of the components of 10 S, we have found that the same preparations investigated after standing more than 3 years in the dry state give precisely the same sedimentation pattern as they did when studied soon after drying.

Recently, Savat[3] (19) has attempted to find some method of producing heavier components from bovine or equine globulins which possess sedimentation constants near 7 S. Many of the bovine γ-globulin preparations were molecularly monodisperse. However, she was unsuccessful in altering the amount of heavy material in some samples by allowing the globulin to stand as an ethanol paste, by drying, or by mild heating. But, since freshly prepared samples of γ-globulin obtained by electrophoretic separation contained only the component of 7 S, she assumed that the component of 10 S is somehow produced during the fractionation procedure. The γ-globulins prepared from various species by Nichol and Deutsch (20) also showed only the components of 7 S.

Some evidence has already been adduced which indicates that the components of 10 S are not fortuitous impurities but are truly γ-globulins. This is particularly indicated by the observations that the same components occur in the various equine and bovine fractions whether obtained from plasma or from milk or colostrum. Immunologically, all of the human γ-globulin fractions cross-react, although particles of different size are represented in the preparations (21).

SUMMARY

1. Bovine immune proteins obtained by chemical fractionation methods from plasma, milk, or colostrum have been examined in the ultracentrifuge. In each instance, the main component (80 to 90 per cent of the total sedi-

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[3] We are grateful to Dr. H. F. Deutsch for lending us a copy of this thesis and permitting us to cite certain of the results.

menting material) has a sedimentation constant of about 7 Svedberg units, and is always associated with a second component of about 10 Svedberg units.

2. The bovine γ-globulins isolated by the electrophoresis-convection method also contain the same two components found in other preparations.
3. Equine γ- and T-globulins containing high tetanus antitoxic activity likewise contain components which sediment at about 7 S and 10 S; larger particles could not be detected in these preparations.
4. The nature of the components of 10 S is discussed.

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ON THE RENEWAL OF THE PURINES OF THE DESOXYPENTOSE AND PENTOSE NUCLEIC ACIDS*

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The nucleic acids fall into two broad categories. The desoxypentose nucleic acids (DNA), in mammalian tissues, are found only in cell nuclei associated with the chromosomes (1, 2). The pentose nucleic acids (PNA) are chiefly cytoplasmic (3), but are also found in smaller amounts in the nucleolus (4) and in the residual chromosomes (5, 1). Various studies have shown that the phosphorus of the desoxypentose nucleic acids is renewed at a slower rate than that of the pentose nucleic acids.

The data presented here indicate that the rate of incorporation of isotopically labeled adenine into the purines of the DNA is extremely slow and, in fact, is not incompatible with the rate of cell mitosis. On the other hand, the purines of the PNA are shown to be in a state of rapid dynamic equilibrium.

Results

The initial observation that labeled dietary adenine was incorporated into nucleic acids, and that it could also serve as a precursor of nucleic acid guanine (6) was made on nucleic acid preparations which were mixtures of the two types of nucleic acids.

A preliminary experiment was then performed (7) in which the PNA and DNA, from the viscera of rats fed isotopically labeled adenine, were fractionated. Adenine and guanine were then isolated from each of the nucleic acid fractions. Isotope analyses (Table III) of each of the purines so obtained revealed that 15.9 per cent and 9.1 per cent respectively of the adenine and guanine of the PNA were derived from the fed material, while only 0.55 per cent and 0.38 per cent respectively of the adenine and guanine of the DNA were derived from the same material. These figures indicate a renewal of DNA purines which is only 3.5 per cent of that of PNA purines.

To determine the extent to which the small amount of isotope found in

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the DNA fraction could have been contributed by contaminating PNA, an experiment was performed in which the effectiveness of the method used to separate the nucleic acids (a modification of the Schmidt-Thannhauser procedure (8, 9)) was tested by means of an isotope dilution technique. A sample of isotopically labeled yeast nucleic acid (10) was added to a sample of rat liver and the mixture was fractionated. The DNA fraction first obtained was found to contain 0.092 atom per cent excess N^{15}, as compared with 2.11 atom per cent excess N^{15} found in the PNA fraction (Table I). These figures indicate that the DNA fraction contained 4.4 per cent PNA as a contaminant. With the expectation of obtaining further purification, the precipitated DNA was redissolved in alkali and was again precipitated by acid. The twice precipitated material so obtained was found to contain only 0.021 atom per cent excess N^{15}, which in turn corresponds to 1 per cent of the isotope concentration present in the PNA fraction. Thus, from the isotope analyses of the first and second DNA fractions it was seen (Table I) that reprecipitation[1] reduced the contamination of the DNA with PNA by a factor of 4. In all succeeding separations of the two types of nucleic acids the DNA was therefore reprecipitated before any further breakdown of this fraction was attempted. In view of the results of this experiment it is apparent that in the experiment on rat viscera, in which a single precipitation of the DNA was employed, a large part of the isotope found in the DNA fraction is to be attributed to the presence of contaminating PNA.

To determine whether the failure of dietary adenine to be incorporated to any extent into DNA was due to the slow renewal of the DNA in resting tissues, or whether, as recently suggested by Reichard (12), this observed failure was due to the fact that adenine cannot be utilized for the synthesis of the purines in DNA, the metabolism of dietary adenine in non-growing and in rapidly regenerating rat livers was studied.

A preliminary experiment showed that, after the feeding of adenine for a single day, the uptake of isotopic nitrogen into the total nucleic acids of different organs varied markedly. It was greatest in the liver (Table II). Isotopically labeled adenine was therefore fed to ten normal adult rats, and PNA and DNA were isolated from the livers alone. After the DNA fraction had been reprecipitated from alkali, total purines, and then adenine, were isolated from it, while adenine and guanine were isolated from the PNA. In non-growing liver, isotope analyses showed DNA and PNA adenine to be derived from dietary adenine to the extent of 0.29 and 21.2 per cent respectively (Table III). If it is assumed that the DNA is un-

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[1] Recently, von Euler, Hevesy, and Solodkowska (11) have used the Schmidt and Thannhauser method with a reprecipitation from alkali. Some decrease in the P^{32} content of the reprecipitated DNA fraction was found there.

TABLE I

Fractionation of Liver Nucleic Acids with Added Labeled Yeast Nucleic Acid (Experiment III)

Nucleic Acid Analyses

	DNA	PNA
	mg.	*mg.*
Estimated in liver sample	33	150
Yeast nucleic acid added	1.4	305
Total hydrolysate	27	432
PNA fraction	Nil	431
Supernatant from 2nd DNA pptn.		5
DNA fraction, twice pptd.	37	Nil

*Isotope Analyses**

	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in purines of PNA
Purines from PNA fraction	2.11	100
DNA, 1st pptn.	0.092	4.4
" 2nd "	0.021	1.0
Copper purines from twice pptd. DNA	0.027	1.3

* Determinations made on Consolidated-Nier mass spectrometer model 21-201; probable error ±0.001 per cent.

TABLE II

Incorporation of Adenine into Mixed Nucleic Acids of Various Organs (Experiment II)

	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed
Dietary adenine	6.30	100
Liver, total purines	0.424	6.7
" adenine	0.549	8.7
" guanine	0.229	3.7
" silver pyrimidines	0.019	0.3
Kidney, total purines	0.301	4.8
Intestine, total purines	0.151	2.4
" adenine	0.285	4.5
" guanine	0.089	1.4
Spleen, total purines	0.105	1.7
Testes, " "	0.003	0.0
Urinary allantoin	1.058	16.8

Table III

Incorporation of Adenine into Nucleic Acids

Experiment No.	DNA						PNA					
	Total purines		Adenine		Guanine		Total purines		Adenine		Guanine	
	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed	Atom per cent excess N^{15}	Calculated on basis of 100 per cent in adenine fed
I. Mixed viscera, adenine fed 10 days			0.035	0.55	0.024	0.38			1.00	15.9	0.57	9.1
IV. Non-growing liver, adenine fed 5 days	0.008	0.17	0.014	0.29					1.02	21.2	0.415	8.7
V. Regenerating liver, adenine fed 5 days	0.419	8.7	0.78	16.3	0.15	3.2	0.566	11.8	1.09	22.7	0.388	8.1
VI. Regenerating liver, 26 days, no supplement after 5 days	0.309	6.5					0.087	1.8	0.132	2.7	0.080	1.7

contaminated by PNA, the renewal of DNA adenine is 1.37 per cent of that of the PNA adenine, or an uncorrected ratio of the PNA to the DNA of 73:1; and if only a quarter of the isotope value of the DNA fraction is attributed to contaminating PNA, the ratio[2] of the renewals of liver PNA purines to DNA purines becomes 100:1.

The same procedures were employed to study the uptake of dietary adenine in the liver nucleic acids of rats during the first 5 days following subtotal hepatectomy. Although the incorporation of dietary adenine into the purines of the PNA of the growing liver (22.7 per cent) was approximately the same as that in non-growing liver, the incorporation into the DNA fraction was enormously increased, and amounted to 16.3 per cent, or 75 per cent of the value found for the purines of the PNA fraction (Table III). This provides evidence that dietary adenine does function as a precursor of the DNA purines when mitosis is occurring and testifies that the small amount of N^{15} incorporated into the DNA purines of non-growing liver must be indicative of an almost negligible renewal of the DNA purines in non-growing tissues.

Additional confirmation of the extremely slow renewal of the DNA purines came from another experiment in which three partially hepatectomized rats were fed isotopic adenine for 5 days and were then continued without further supplement of adenine for 21 days. At the end of this period the isotope value (0.309) of the total DNA purines obtained from the liver was not much lower than the 5 day value (0.419) of the previous experiment. In contrast, the isotope values of the PNA purines had fallen markedly, indicating that they had been extensively replaced by non-isotopic precursors during this period (Table III). In accord with available data on the growth of regenerating rat liver (15–17), the livers removed on the 6th day amounted to 85 per cent of the estimated original liver weight, and those removed on the 27th day amounted to 108 per cent of the original weight. If allowance is made for this additional growth utilizing non-isotopic precursors between the 6th and the 27th days, nearly all of the decrease in the isotope value of the DNA purines is accounted for.

EXPERIMENTAL

Feeding and Maintenance of Experimental Animals—The rats used in all experiments were adult, male, Sherman strain animals averaging 300 gm. in weight. They were housed in individual metabolism cages and were kept on a diet of pulverized, moistened Purina chow pellets. The adenine, labeled in the 1 and 3 positions with isotopic nitrogen (6, 18) was admixed with this diet, and the daily allotment of food was adjusted so that each animal received 0.2 mM of adenine (except in Experiment II; 0.4 mM) and 50 gm. of chow (dry weight) per kilo of body weight per day. On the afternoon of the day following the last feeding the animals were sacrificed either by decapitation or by injection of urethane followed by injection of magnesium sulfate. The organ or organs to be worked up were quickly removed and frozen on dry ice. They were stored at −30° until further treatment.

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[2] Nitrogen from biological sources contains about 0.005 atom per cent more N^{15} than does commercial tank nitrogen (13, 14) and the values are all reported here as atom per cent excess over tank nitrogen. The subtraction of 0.005 from each value would result in an appreciable increase in the ratios.

Subtotal Hepatectomy Technique (*15*)—Adult rats, without any previous treatment, were anesthetized with ether and the left lateral and both central lobes of the liver were resected $\frac{1}{4}$ inch distal to the ligature. A small amount of sulfadiazine was sprinkled on the wound. Feeding was commenced on the day following the operation.

Fractionation of DNA and PNA from Tissues—A modification of the Schmidt-Thannhauser procedure (8) was used in all experiments as follows: The weighed, frozen tissues were homogenized with 10 volumes of water in a Waring blendor at 5°, and an equal volume of cold, 14 per cent trichloroacetic acid was added to the homogenate. The resulting mixture was stirred for half an hour, and was then centrifuged in 250 cc. centrifuge tubes. The residue was washed by centrifugation, once with 1 per cent trichloroacetic acid, then with water, with anhydrous ethanol, and finally with ether. All operations were carried out at 5°. The tissue powder was then defatted by hot extraction for 2 hours with 1:1 methanol and chloroform. The desiccated tissue was placed in a rubber-stoppered Erlenmeyer flask with 1 N KOH (10 cc. per gm. of fresh tissue), and incubated at 37° for 20 hours, after which time all the tissue was in solution. After small aliquots were removed for DNA (19) and PNA (20, 21) analyses, the DNA fraction was precipitated from the total hydrolysate by addition, with rapid stirring and ice cooling, of one-tenth the volume of 25 per cent trichloroacetic acid, and enough 6 N HCl to make the solution 0.1 N with respect to HCl. When precipitation was complete, most of the supernatant was removed through a filter stick, and the precipitated DNA fraction was finally collected by centrifugation. The supernatant (PNA) was filtered through Celite to eliminate last traces of suspended solids, and an aliquot was removed for analysis. The solids were washed once with 2.5 per cent trichloroacetic acid, centrifuged, and the supernatant discarded. In all but the rat viscera experiment, the DNA fraction was redissolved in 1 N KOH (1 cc. per gm. of fresh tissue) by stirring at room temperature for 20 minutes, and then reprecipitated with proportionate amounts of trichloroacetic and hydrochloric acids. The DNA was then extracted from the total residue with 5 per cent trichloroacetic acid (0.5 cc. per mg. of DNA estimated to be present) by heating with stirring at 90° for 20 minutes. The extract was separated by centrifugation, and the residue was then reextracted with one-half the previous volume of 5 per cent trichloroacetic acid. The extracts were combined, filtered to eliminate last traces of solids, and an aliquot was removed for analysis.

Isolation of Purines from DNA and PNA Fractions—Purines were isolated from the two nucleic acid fractions as either the copper or the silver salts.

To isolate purines in the form of their copper salts from a PNA fraction, the filtered supernatant was used as such; to isolate copper purines from DNA, the hot trichloroacetic acid solution of the DNA was used. In either case 10 N H_2SO_4 was added to make the solution 0.4 N with respect to H_2SO_4, and hydrolysis was accomplished by refluxing for 2 hours. It was cooled, adjusted to pH 9 with NaOH, and then to pH 5 with dilute H_2SO_4. It was then reheated in a water bath, and the copper purines were precipitated with $NaHSO_3$ and $CuSO_4$ according to Hitchings (22). The solution was vigorously stirred throughout the course of the addition of reagents. The supernatant was largely removed through a filter stick, and the precipitated copper purines were collected and washed twice with hot water by centrifugation. The washed material was dissolved in a minimal amount of hot 3 N HCl, copper was removed by precipitation with H_2S, and the CuS was washed with hot dilute HCl. Last traces of H_2S were removed from the combined filtrate and washings by aeration. Guanine was then allowed to precipitate at about pH 6, and adenine was isolated as the picrate from the supernatant (23). The guanine was crystallized as the sulfate from 1 N H_2SO_4 and recrystallized one or more times. The adenine picrate was twice recrystallized from 25 per cent acetic acid. A portion of the adenine picrate was suspended in 0.1 N HCl and, after continuous extraction with ether, the solution of adenine hydrochloride so obtained was used for isotope analysis.

The same PNA and DNA starting materials were used for the isolation of silver purines as were used for isolation of the copper salts. Enough 6 N HCl was added to the solution of the fraction to make it 1 N in HCl, and it was refluxed for 1 hour. The silver purines were precipitated with an excess of ammoniacal silver chloride (24). They were collected by decantation, washed several times with water, and converted to the purine hydrochlorides by treatment with 25 and 10 cc. portions of 3 N HCl (25). The purine hydrochloride solution was filtered through Celite, and guanine and adenine were separated and purified. All final products were analyzed for nitrogen content by the micro-Kjeldahl method.

Preparative Separation of Adenine and Guanine by Counter-Current Dis-

tribution—The purines were recovered from 15 to 30 mg. samples of copper purines by two treatments with 1 N HCl and H_2S in a micro hydrogenation apparatus. The purine hydrochloride solution was concentrated to dryness and dissolved in a few drops of 2 N NaOH and 8 cc. of buffer.

Adequate separation of 5 to 10 mg. quantities of mixtures of adenine and guanine was accomplished in a system of 0.1 M potassium phosphate, pH 9.5, and *n*-butanol-isopropanol (60:40). In this system the spread between the distribution constants (guanine 0.5, adenine 1.3) is not as great as in the system at pH 6.5 (26), but several times as much guanine may be dissolved in the alkaline system.

Thirty-two transfers, without withdrawals, were made in a twenty-five tube counter-current distribution apparatus (27), at which point the leading edge of the adenine band has just begun to overlap the guanine band. With 5 mg. quantities of adenine in Tube 0 a symmetrical curve is obtained. 5 mg. of guanine yield an unsymmetrical curve, the leading side of which gives evidence of a K corresponding to that obtained with dilute solutions of guanine, but the K for the trailing side is lower than expected, due to inadequate solubility in the upper phase which prevents attainment of an equilibrium between the two phases until after several extractions of Tube 0 have been made. From the patterns obtained from mixtures the leading half of the adenine band and the trailing half of the guanine band were selected. To avoid dilution by extraneous nitrogen it was necessary to concentrate the contents of the selected tubes to about one-quarter volume and to precipitate the copper adenine or copper guanine.

Control experiments with mixtures of isotopic guanine and non-isotopic adenine, and vice versa, showed cross-contaminations of 0.0 to 0.3 per cent of guanine in the adenine fractions selected and 1 to 2 per cent of adenine in the guanine fractions.

In the preparation of the PNA purines from Experiment VI the CuS supernatant, as introduced into Tube 0, contained 0.086 per cent N^{15} (copper purines 0.087); the reasonably pure guanine from Tubes 4 through 8 contained 0.080; the mixture from Tubes 10 through 16 contained 0.110; and the pure adenine from Tubes 17 through 24 contained 0.132. In addition the contents of Tubes 0 through 3, which contained a trace of adenine and considerable guanine, were precipitated and analyzed and were found to contain only 0.040 per cent N^{15}, which indicates the presence of non-isotopic nitrogen in a form possessing a low distribution constant in this solvent pair, possibly cysteine or glutathione. The contamination of copper purine preparations with non-purine nitrogen is not unexpected (22) when these precipitates are prepared directly from an alkaline digest of tissue, as was the case with the copper purines from the PNA fractions. The possible presence of contaminants not absorbing at 260 mμ and having distribution constants similar to either of the purines must also be recognized, and the isotope values determined directly on the copper purine preparations must be considered as minimal values.

Experiment I. Incorporation of Dietary Adenine into DNA and PNA of Pooled Rat Viscera—Five rats totaling 1510 gm. in weight were used. They were fed adenine containing 6.3 atom per cent excess N^{15} (6) for 10 days. The thymus, lungs, heart, spleen, kidneys, liver, stomach, testes, and large and small intestine of each animal were pooled and utilized. A small sample of the defatted tissue was removed for PNA and DNA analyses. The DNA fraction was precipitated only once. Total purines were isolated as the copper salts. All final products were characterized by counter-current distribution (26). In this experiment an inadequate quantity of guanine was obtained from the DNA fraction and it was characterized only by counter-current distribution. Isotope values of the isolated products are given in Table III.

Analysis—Total mg. of nucleic acid in the following: desiccated tissue, DNA 560, PNA 749; total hydrolysate, DNA 314,[3] PNA 836; DNA fraction, DNA 400, PNA nil; PNA fraction, DNA nil, PNA 781.

DNA fraction; adenine picrate, $C_5H_5N_5 \cdot C_6H_3O_7N_3 \cdot H_2O$, calculated, N 29.3; found, N 28.9.

PNA fraction; adenine picrate, calculated, N 29.3; found, N 28.9; guanine, $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot H_2O$ (dried at 130°), calculated, N 33.4; found, N 34.2; $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$ (rehydrated and dried at 20°), calculated, N 32.1; found, N 32.3.

Experiment II. Incorporation of Adenine into Total Nucleic Acids of Various Rat Organs—Eight rats, weighing a total of 2516 gm., were fed adenine (6.30 atom per cent excess N^{15}) at a level of 0.4 mM (54 mg.) per kilo of body weight per day for a single day. The pooled livers, kidneys, spleens, testes, and intestines were individually worked up for total nucleic acids (6). In the cases of the livers and intestines sufficient nucleic acids were available to permit the separation of adenine and guanine, but from the other organs copper purines only were prepared. Allantoin was isolated from the pooled urines. The isotope concentrations of all final products are given in Table II.

Experiment III. Fractionation of Liver Nucleic Acids with Added Labeled Yeast Nucleic Acid—17.2 gm. of normal rat liver, estimated to contain 150 mg. of PNA and 33 mg. of DNA, were worked up as usual but with the addition of 305 mg. of purified yeast nucleic acid (containing 3.35 atom per cent excess N^{15}) (10) just before hydrolysis in 1 N KOH. The solution containing once precipitated DNA, as well as the trichloroacetic acid extract containing twice precipitated DNA, was subjected to isotope analysis. Copper purines were isolated from the PNA fraction and from the twice precipitated DNA fraction. The results of nucleic acid analyses and of isotope determinations on all final and intermediate products are indicated in Table I.

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[3] Low values were always obtained on this fraction, presumably due to interference by other constituents of the hydrolysate.

Experiment IV. Incorporation of Adenine into PNA and DNA of Non-Growing Rat Liver—Ten rats, totaling 3200 gm., were fed adenine (4.80 atom per cent excess N^{15}) (18) for 5 days. A total of 87 gm. of liver tissue, representing 2.7 per cent of the total body weight, was removed at autopsy. The pooled livers were worked up as described above. All purines were isolated as the silver salts. Insufficient guanine was obtained from the DNA fraction to be able to prepare an adequately purified sample. Results of isotope determinations are indicated in Table III.

Analysis—Total mg. of nucleic acid in the following: desiccated liver, DNA 140, PNA 768; once precipitated DNA, DNA 117, PNA 38;[4] twice precipitated DNA, DNA 165, PNA nil; PNA fraction, DNA nil, PNA 760.

DNA fraction; adenine picrate, $C_5H_5N_5 \cdot C_6H_3O_7N_3 \cdot H_2O$, calculated, N 29.3; found, N 29.5.

PNA fraction; adenine picrate, calculated, N 29.3; found, N 30.2; guanine, $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$, calculated, N 32.1; found, N 32.5.

Experiment V. Incorporation of Dietary Adenine into PNA and DNA of Regenerating Rat Liver—Seventeen rats were partially hepatectomized. Starting on the day following the operation, and continuing for 5 days thereafter they were fed adenine (containing 4.80 atom per cent excess N^{15}), and were sacrificed on the day following the last feeding. In the strain and weight class of rats used in these experiments we have found that the intact liver averages 2.7 per cent of the total body weight. The total weight of all rats employed in this experiment was 4880 gm. and the total weight of their intact livers was therefore estimated to be approximately 133 gm. Of this, 89.9 gm., or 67 per cent (range 54 to 80), were removed at operation. A total of 113.7 gm., or 85 per cent (range 71 to 117) of the estimated original weight, was obtained at autopsy. This leads to an estimate that, of the liver tissue used in this experiment, approximately 62 per cent by weight was newly formed during the 6 days following the operation.

The regenerated livers obtained at autopsy were treated as were those obtained in the experiment with normal liver. Part of each nucleic acid fraction obtained was used for the preparation of copper purines, the isotope content of which is reported under total purines. Silver purines were isolated from the remainder of each fraction. Adenine and guanine were isolated from the silver purines of the PNA, but an attempt to isolate these compounds from the silver purines of the DNA was unfortunately unsuccessful. In order to obtain DNA adenine and guanine for isotope determinations, a portion of the DNA copper purines was separated by means of the counter-current distribution procedure. Essentially pure adenine was obtained in this way, but the guanine fraction obtained was estimated to be contaminated with adenine to the extent of possibly 10 per cent. The results of isotope determinations on all products are given in Table III.

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[4] This unexpectedly high value is possibly due to analytical error.

Analysis—Total mg. of nucleic acid in the following: total hydrolysate, DNA 176, PNA 1312; twice precipitated DNA, DNA 225, PNA nil; PNA fraction, DNA nil, PNA 1321.

PNA fraction; adenine picrate, $C_5H_5N_5 \cdot C_6H_3O_7N_3 \cdot H_2O$, calculated, N 29.3; found, 30.0; guanine, $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$, calculated, N 32.1; found, N 32.7.

Experiment VI. Retention of Isotopic Adenine Once Incorporated into PNA and DNA of Regenerating Rat Livers—Three rats, totaling 836 gm., were partially hepatectomized, and 18.6 gm. of liver were removed. After being fed adenine (4.80 atom per cent excess N^{15}) for 5 days, the animals were maintained on an unsupplemented diet for an additional 21 days. They were sacrificed on the 27th day after operation, and 24.7 gm. of liver tissue were obtained at autopsy. This represents 108 per cent (range 103 to 111) of the estimated original weight, and 83 per cent of this weight is estimated to have been formed during the 26 day period. The tissues were worked up as usual, and copper purines were isolated from both the PNA and DNA fractions. Adenine and guanine were separated from the PNA copper purines by counter-current distribution, but since only about 4 mg. of purines were present in the total DNA fraction, no attempt to separate them was warranted. Isotope analyses of final products are given in Table III.

Analysis—Total mg. of nucleic acid in the following: total hydrolysate, DNA 31, PNA 200; twice precipitated DNA, DNA 41, PNA nil; PNA fraction, DNA nil, PNA 180.

DISCUSSION

The several studies of nucleic acid phosphorus metabolism, which have recently been reviewed by Hevesy (28), have shown that the rate of renewal of the phosphorus of the DNA fractions is slower than that of the PNA fractions. However, none of the work with P^{32} has shown so marked a difference between the rates of replacement of the PNA and the DNA phosphorus as has been observed here for the PNA and DNA purines. Hammarsten and Hevesy (29) found that for the total rat the ratio of the turnovers of PNA phosphorus to DNA phosphorus was 1.66:1 after 2 hours, and for individual organs was 33:1 in the liver, 2:1 in the intestine, and 3:1 in the spleen. The ratio of PNA phosphorus to DNA phosphorus of 33:1 for liver is the only one even approaching the uncorrected ratio of 73:1 which we have observed for the relative incorporation of adenine into the PNA and DNA purines. None of the phosphorus figures on total viscera are of a comparable magnitude.

The differences between the relative renewals observed with adenine and with phosphate might be explained by the replacement of a small amount of the DNA phosphorus when no change was occurring in the carbon-nitrogen skeleton of the molecule.

Marshak (30) has recently demonstrated that, after the administration of P^{32}, no significant amount of P^{32} is liberated by the action of desoxyribonuclease from nuclei isolated from non-growing tissue, while much P^{32} is liberated by the action of ribonuclease. The histological localization of newly incorporated P^{32} in the DNA of tissues of high mitotic rate has recently been demonstrated by radioautographic methods (31).

Brues, Tracy, and Cohn (32), using a different method for the separation of the two types of nucleic acids, found that in rat liver the ratio of turnovers of PNA phosphorus to DNA phosphorus was 5.2:1 on the 3rd day after and 5.9:1 on the 8th day after a single injection of P^{32}. Brues has commented (33) that in subsequent experiments higher ratios have been obtained. These authors found that in regenerating liver there was a proportionately greater incorporation into, and a longer retention of, the phosphate of the DNA fraction.

When isotopic ammonia was fed (34), a negligible uptake was obtained in the desoxypentose nucleic acids, but the isotope values encountered were so low that a reliable ratio of the rates of renewal could not be calculated. Hammarsten and coworkers (35) have shown that the nitrogen of N^{15}-labeled glycine is incorporated into the DNA purines of rat livers at a slower rate than into the PNA purines and that the PNA fraction associated with isolated nuclei is renewed more rapidly than is the cytoplasmic PNA fraction. It was also shown that in regenerating liver the purines of the DNA fraction show an increased uptake of the nitrogen of the glycine, a picture generally comparable to the one found with adenine. However, in the experiments with glycine, the renewal of purine nitrogen in the DNA fraction of non-growing liver was considerable, and the ratio of the rates of renewal of the purine nitrogen of the total liver PNA fraction and the DNA fraction was 4:1, a ratio of an entirely different magnitude from that found with adenine.

It was also found (35) that with glycine the incorporation into the purines of the PNA of regenerating liver was much greater than into the PNA purines of non-growing liver. On the other hand, in these experiments with adenine, the incorporation into the PNA purines of non-growing or of regenerating liver was essentially identical. Rittenberg, Sproul, and Shemin (36) have found that the incorporation of glycine into the proteins of non-growing or into regenerating liver was at about the same rate and they concluded that growth was the result of the inhibition of degradative reactions rather than the increase of the synthetic processes. The same interpretation could be applied to the results obtained here for adenine incorporation into the PNA.

In the data reported here the relative introduction of N^{15} of the dietary adenine into the nucleic acid guanine and the nucleic acid adenine has not been as constant as it appeared at first (37). In the original experiments involving the total nucleic acids of mixed viscera, the relative incorporation of the N^{15} into the two purines was in the ratio of 0.60 and 0.59 (6), while in the present experiment with mixed viscera it was 0.57 in the purines of the PNA fraction. However, in the total nucleic acids of individual organs the ratio of the isotope in the guanine to that in the adenine was 0.31 for intestine and 0.42 for liver. In the separated PNA fractions, it was 0.41 for the non-growing liver and 0.36 for the regenerating liver.

An estimate may be made of the biological half life of the purines of the PNA fraction from the decrease in the isotope concentration between the 6th and 27th days (Experiments V and VI). The isotope value for the copper purines fell in 21 days from 0.566 to 0.087, or to 15 per cent of its initial value. If the loss of N^{15} follows a first order course, the half life computed from these values is nearly 8 days, while that computed from the corresponding adenine values is 7 days, and from the guanine values is 9 days. If allowance is made for the growth occurring between the 6th and 27th days, a somewhat longer half life is indicated. It is interesting that this biological half life of about 8 days for the purine ring nitrogen of the combined pentose nucleic acids of liver in the latter stages of regeneration is of approximately the same order as that of the protein nitrogen of non-growing liver, which was shown by Shemin and Rittenberg (38) to be 7 days. This dynamic state of the pentose nucleic acids may well be correlated with a functional rôle in the metabolic processes of the cell.

The lowest values for the absolute rate of renewal of the phosphorus of DNA, calculated from the P^{32} value of the inorganic phosphate, have indicated that a replacement of about 0.1 per cent of the DNA phosphorus of the liver or kidneys occurs in a 2 hour period (29), or a rate of 1 per cent per day (39), a rate which could not be accounted for solely by the new DNA arising through mitosis (28). In the case of nucleic acid adenine, it is not possible to determine the concentration of the isotope in the direct precursor, since the relative contributions of the labeled exogenous adenine and non-labeled material elaborated *in vivo* are unknown. However, from the estimated half life of the PNA purines of about 8 days, it may be calculated that 8.5 per cent renewal would occur per day, or about 36 per cent in 5 days. From this value and the observed ratio of the rates of renewal of the PNA and DNA purines it may be estimated that, if the proportions of isotopic and non-isotopic precursors of the two types of nucleic acids are the same, the maximum renewal of the DNA purines in non-growing tissue is of the order of 0.4 per cent in the 5 day period. This is appreciably less than the renewal rate for the phosphorus, and a value not incompatible with available estimates of the rate of mitosis occurring in non-growing liver (17, 28, 35, 40).

Thus, it is conceivable that all of the isotope incorporated into the DNA purines was introduced in the process of formation of new nuclei, and that, once it is formed, the skeleton of the DNA is not at all in dynamic equilibrium with other constituents of the cell. This can be correlated with the cytological observation of Ris (41) that the chromosomal components double in the course of mitosis, and the recent report by Vendrely and Vendrely (42) that, within a species, chemical analyses indicate a constant DNA content per nucleus and that the amount of DNA in haploid nuclei is one-half that in diploid nuclei. Inhibitors of desoxyribonuclease, such as those recently reported (43, 44), may function to maintain this stability of the DNA throughout the interphase period of the cell.

The significance of the fact that the desoxypentose nucleic acids are an exception to the general concept of the rapid dynamic equilibrium of body constituents (45) is not obvious, but this biochemical stability furnishes a desirable (46) permanence which adds to the circumstantial evidence that desoxyribonucleic acid may be a fundamental component of the gene.

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SUMMARY

In both total viscera and in non-growing liver of rats it has been shown that isotopically labeled dietary adenine is rapidly incorporated into the purines of the pentose nucleic acid fraction, but that the incorporation into the purines of the desoxypentose (nuclear) nucleic acid fraction is about 1 per cent as extensive.

During the period of rapid regeneration of the liver of partially hepatectomized rats it was found that the incorporation of purines into the des-

oxypentose nucleic acids was about 75 per cent of that into the pentose nucleic acid fraction. When similarly treated animals were then allowed an unsupplemented diet for 3 weeks, the purines of the pentose nucleic acids were largely replaced by non-isotopic precursors, while the purines of the desoxypentose nucleic acids were replaced to but a small extent.

An isotope dilution analysis of the method used for the separation of the two classes of nucleic acids has been carried out.

A survey of the incorporation of dietary adenine into several organs of the rat is reported.

The extent of incorporation of dietary adenine into the desoxypentose nucleic acids of liver can be roughly correlated with the extent of cell mitosis, while the dynamic state of the purines of the pentose nucleic acids of liver is comparable to that of the protein of liver.

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THE DIRECT OXIDATION OF ADENINE IN VIVO*

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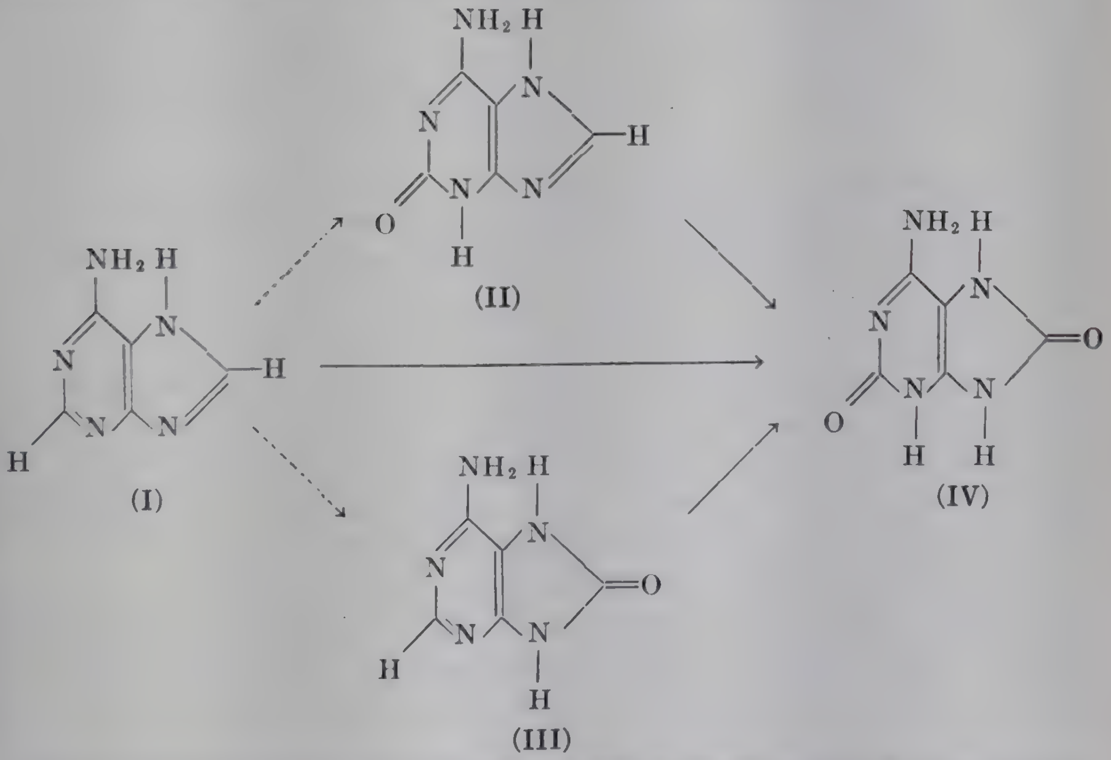
When large quantities of adenine are administered to various mammalian species, extensive crystalline masses, which for some time were mistaken to be uric acid (1), deposit in the kidneys (1–3). Nicolaier (2) believed the isolated transformation product to be 2,8-dioxyadenine (4), and this was confirmed by Fischer (2), although the experimental data leading to the elucidation of the structure were never reported. Interest in this problem was renewed 48 years after the initial observation by the report that large amounts of adenine produce a rise in blood pressure and a syndrome resembling avitaminosis (blacktongue) in dogs (5) and a slight increase in the rate of growth of sarcoma 180 in mice (6).



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† Postdoctoral fellow of the National Cancer Institute.

We have studied the fate of large doses of adenine, administered either orally or intraperitoneally (Table I), and have observed the deposition of these crystals in kidneys. A rigorous comparison of synthetic 2,8-dioxyadenine with the natural product from the kidneys of rats which had received adenine was made (Table II).

An examination of the structural formula of adenine (I) reveals that the carbon atoms at positions 2 and 8 are both amidine carbon atoms, and it would be difficult to decide *a priori* what the course of the oxidation to 2,8-dioxyadenine (IV) might be. Since 2-oxyadenine (isoguanine) occurs naturally in butterfly wings (7) and as the 9-D-riboside (crotonoside) in croton beans (8, 9), whereas 8-oxyadenine has not been found in nature, it appeared reasonable that the conversion of I to IV might occur via a prior oxidation in the 2 position.

Accordingly, isoguanine (II) (10) was tested and it was found, indeed, to serve as a precursor of 2,8-dioxyadenine (IV). However, we have found that the administration of 8-oxyadenine (III) (11) also gives rise to clusters of 2,8-dioxyadenine (IV) crystals in rat kidney. Thus, it seems that, so far as these transformations are concerned, extensive oxidation of adenine can proceed in the 2 or 8 position, irrespective of whether the other position is substituted by oxygen. This is reminiscent of the behavior of hypoxanthine, xanthine, and 6,8-dioxypurine towards xanthine oxidase from milk or ox spleen; in all three cases, uric acid was the final product (12).

Of more immediate pertinence is the report by Booth (13) of the oxidation of adenine by xanthine oxidase from whey. It was found that a product responding to the Benedict uric acid test resulted from the action of this enzyme on adenine. However, since no ammonia was evolved and 2 atoms of oxygen were consumed per molecule of adenine oxidized, it appears likely that the product actually obtained was 2,8-dioxyadenine. The enzyme was active against 8-oxyadenine and somewhat less so against isoguanine; in both cases 1 atom of oxygen per molecule was consumed and no ammonia was evolved, and the products gave a positive Benedict uric acid test. The lack of specificity of the Benedict test is well known,[1] and it is probable that 2,8-dioxyadenine was the final product.

In order to determine whether any of the 2,8-dioxyadenine deposited in the kidney had arisen from "endogenous" adenine of the animal in addition to that administered, adenine, containing 0.492 atom per cent excess N^{15} (labeled in the 1 and 3 positions (14)), was given orally to rats at a level of 500 mg. per kilo. It was found that the 2,8-dioxyadenine isolated from the kidneys contained 0.473 atom per cent excess N^{15}. From this dilution, it may be calculated that about 20 mg. of adenine per kilo of rat tissue were available, during the 17 hours of the experiment, for admixture with the labeled dietary adenine, participating with the latter in 2,8-dioxyadenine formation.

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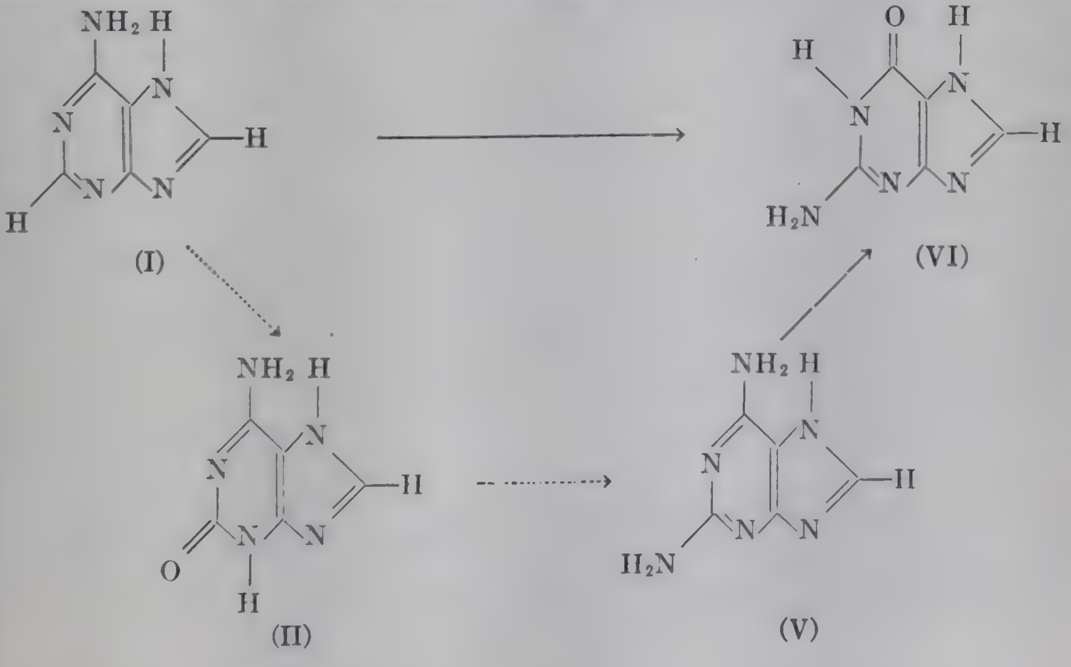
[1] Dr. T. F. Yü and Dr. A. B. Gutman, Columbia Research Service, Goldwater Memorial Hospital, New York, have had the kindness to determine the chromogenic properties of 2,8-dioxyadenine with the Benedict reagent. It was found that 2,8-dioxyadenine gives almost as much color as does uric acid, and that 2,8-dioxyadenine is not acted upon by uricase.

In another experiment, isoguanine, containing 1.51 atom per cent excess N^{15} (10), was given to rats by the intraperitoneal route. The 2,8-dioxyadenine, isolated from the kidneys, contained 1.48 atom per cent excess N^{15}. It is not necessarily valid to argue from these figures that isoguanine is normally present in the tissues of the rat. The dilution observed in this case is less than that obtained when adenine was given, but it does indicate that a small amount of a precursor leading to 2,8-dioxyadenine is normally present in rat tissue. The observed conversion of isoguanine to 2,8-dioxyadenine is over 20 times the conversion to allantoin, which was determined in conjunction with an isotope dilution analysis of the urinary allantoin.

The isotopic isoguanine experiment afforded an opportunity to investigate, in part, the mechanism whereby adenine is converted to guanine in the rat (15). A reasonable scheme (16) involves the oxidation of adenine (I) in the 2 position to give isoguanine (II). The possibility of such an oxidation is indicated by the studies described here. The next step would then be an amination of the 2-hydroxyl to give 2,6-diaminopurine (V), a compound which is known to be utilized for the biosynthesis of nucleic acid guanine (VI) (17). From the data in Table III, it is seen that iso-



guanine is not utilized by the rat for nucleic acid synthesis. Perhaps the rapid oxidation in the 8 position to yield 2,8-dioxyadenine precludes any significant rôle it might otherwise play in nucleic acid synthesis in the rat.

When the extremely limited solubility of 2,8-dioxyadenine[2] is taken into consideration, it is not surprising to find kidney deposits of this compound following administration of its precursors in large amount. This process results in renal damage which, if sufficiently severe, may cause uremia and consequently secondary changes in other organs. Current studies of adenine intoxication in rats show that an anemia, resembling the anemias observed in advanced renal diseases in man (18), can develop following severe renal damage.[3] The origin of the hypertension and a syndrome resembling "avitaminosis" (blacktongue) in dogs (5) might have to be reconsidered in this light. The results of an investigation of the circumstances under which 2,8-dioxyadenine deposits in kidney, causing a condition we have termed "adenine kidney," will be presented elsewhere.[3]

It is remarkable that no 2,8-dioxyadenine could be demonstrated in the kidney after it was administered, either orally or intraperitoneally. That this failure to deposit in the kidney is not due to lack of absorption from the peritoneal cavity is demonstrated by the complete absence of 2,8-dioxyadenine in the peritoneal fluid 1 day after its injection. Extensive degradation must have occurred, since none could be demonstrated in the tissues and only trace amounts were found in the urine. The administration of isotopically labeled 2,8-dioxyadenine will be necessary to elucidate its fate.

EXPERIMENTAL

"Adenine Kidney"—Macroscopically, kidneys of animals given adenine, isoguanine, or 8-oxyadenine by the oral or intraperitoneal route were enlarged, pale, and, in advanced stages, yellow-stippled and granular on the surface. In a cross-section, cortex and midzone were seen to be swollen. Microscopically, crystalline deposits, consisting of rosettes of yellow needles, were found in distal tubules, which appeared to accumulate proximally into Henle's loops and, in advanced cases, even into proximal tubules. Following administration of 2,8-dioxyadenine, only a few isolated bodies, the size of red cells, and staining bluish with hematoxylin, were found in distal tubules. Details of finer histological lesions will be reported separately.[3]

Oral Administration of Isotopic Adenine—Two male rats, weighing a total of 398 gm., received by stomach tube 190 mg. of adenine, containing 0.492 atom per cent excess N^{15} (14), prepared as a 2.5 per cent solution in 0.4 N lactic acid. The animals, kept on a Rockland stock diet (complete), were sacrificed 17 hours later, and the kidneys, which were much enlarged and showed typical "adenine kidney" lesions, were homogenized in dry ice-alcohol and dried with alcohol and ether. The kidney powder was extracted for 15 minutes at 100° with 15 ml. of 2 N HCl. The extract was neutralized with concentrated ammonia, and the precipitate was collected after chilling. The crude 2,8-dioxyadenine was purified by dissolving in hot 2 N HCl and then precipitating with concentrated ammonia; 32 mg. were obtained, which amounted to about a 14 per cent conversion from the ingested adenine. After three more reprecipitations, the 2,8-dioxyadenine, dried at room temperature in a vacuum desiccator over P_2O_5, was found to contain 0.473 atom per cent excess N^{15}.

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[2] The solubility of 2,8-dioxyadenine hemihydrate was found to be about 2 mg. per liter of water at room temperature. In comparison, uric acid has a solubility of about 55 mg. per liter.

[3] Philips, F. S., Thiersch, J. B., and Bendich, A., in press.

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$. Calculated, N 39.8; found, N 39.9

The ultraviolet spectrum was that of 2,8-dioxyadenine (11) (see Table II). From the dilution from 0.492 to 0.473 atom per cent excess N^{15} it may be calculated that there were about 20 mg. of free adenine available per kilo of body weight which had mixed with the ingested isotopic adenine.

Intraperitoneal Administration of Isotopic Adenine—Eight rats with an aggregate weight of 1520 gm. received for 4 days daily intraperitoneal injections of isotopic adenine (prepared as described above). A total of 528 mg. of adenine was injected. The animals were sacrificed on the 5th day. There was a considerable accumulation of ascitic fluid in the peritoneal cavity; otherwise the kidneys presented the same picture described above. The kidneys were processed as above and yielded 52 mg. of 2,8-dioxyadenine which contained 0.461 atom per cent excess N^{15}. A sample was recrystallized from 2 N H_2SO_4.

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$. Calculated, N 39.8; found, N 39.7

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2SO_4 \cdot \frac{1}{2}H_2O$. Calculated, N 31.1, S 7.12; found, N 31.6, S 7.22

The remaining organs were pooled and were homogenized and dried with alcohol and ether. Sodium nucleate was prepared from the tissue powder. Adenine and guanine were isolated from the sodium nucleate according to procedures previously described (15). Urea, ammonia, and allantoin were isolated from the urine. The N^{15} content of the various constituents is listed in Table I.

It was possible to demonstrate considerable amounts of free adenine in the urine by means of filter paper chromatography in several media. No 2,8-dioxyadenine, isoguanine, or 8-oxyadenine was seen, although by this technique a few micrograms of these compounds can be detected.

Comparison of Synthetic and Natural 2,8-Dioxyadenine—The identity of synthetic (11) and natural 2,8-dioxyadenine (from rat kidney) was established by determining the elementary composition, by comparing the ultraviolet absorption spectra and the mobility of samples on filter paper strips. As for the filter paper chromatography, the procedures and solvent systems described by Vischer and Chargaff (19) were utilized. The "spots" were made visible both by the HgS method (19) and by exposure to an ultraviolet lamp (Mineralight).[4] In all cases, the "spots" coincided by both methods and revealed the presence of single components only. R_F values were computed by the usual procedure (20).

The comparison is given in Table II. The slight discrepancies in the E_M values listed in Table II are well within the experimental error encountered in such measurements with the Beckman quartz spectrophotometer, model DU (21).

TABLE I

Administration of Isotopic Adenine

Adenine, 0.492 atom per cent excess N^{15}.*

	Intraperitoneal	Oral
	atom per cent excess N^{15}	*atom per cent excess N^{15}*
2.8-Dioxyadenine, from kidney	0.461	0.473
Sodium nucleic acids	0.026	
Free nucleic acids	0.026	
Adenine	0.054	
Guanine sulfate	0.024	
Silver pyrimidines	0.004	
Ammonia	0.011	
Urea	0.008	

* Consolidated-Nier ratio mass spectrometer, model 21-201; probable error ±0.001.

Solubility of 2,8-Dioxyadenine in Water—About 5 mg. of 2,8-dioxyadenine hemihydrate were shaken at 23° with 10.0 ml. of water for 17 hours. The mixture was filtered and the filtrate was diluted with an equal volume of 2 N HCl. From the optical density (0.098) and the E_M of 17,700, it was calculated that the solubility of 2,8-dioxyadenine hemihydrate was about 2.0 mg. per liter of water.

To safeguard against the possibility that equilibrium might not have been reached, the experiment was repeated with these modifications. The suspension was heated for 15 minutes at 100° and then shaken at 25°

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[4] We are indebted to Dr. C. E. Carter, Oak Ridge National Laboratory, for placing this method at our disposal prior to publication.

for 2 days. The solubility was found to be about 2.2 mg. per liter of water.

Effect of Intraperitoneal Administration of Isoguanine (2-Oxyadenine)—Four rats, with an aggregate starting weight of 723 gm., were given daily injections by the intraperitoneal route for 3 days a total of 237 mg. of isoguanine (10) (0.5 per cent solution in 10 per cent gum acacia made up in 1 per cent NaCl, and containing 2 molar equivalents of lactic acid). The dose was equivalent to 100 mg. of adenine per kilo. The animals were sacrificed 1 day later, and their kidneys were removed. The peritoneal cavity contained considerable sterile ascitic fluid. The fluid was mixed with an equal volume of 2 N HCl, and the mixture was heated at 100° for 15 minutes and then filtered. The filtrate showed only non-specific ultraviolet absorption. Typical "adenine kidneys" were found. No other significant tissue changes were observed on microscopic examination. After freezing in dry ice-alcohol, the kidneys were homogenized in the Waring blendor and dried. The residue was extracted with 12 ml. of 2 N HCl at 100° for 20 minutes. The hot extract was neutralized with concentrated ammonia, cooled overnight, and the precipitate was collected. It was redissolved in acid, treated with norit, and reprecipitated. 75.1 mg. of a colorless solid, which amounted to a conversion of 26 per cent, were obtained. The preparation was recrystallized four times in this fashion and dried at room temperature in a vacuum desiccator over P_2O_5.

TABLE II

Comparison of Synthetic and Isolated 2,8-Dioxyadenine

Solvent	Wave-length	Ultraviolet spectrum, E_M	
		Synthetic	From kidney
	mμ		
0.1 N phosphate, pH 6.8	235	8,890	8,950
0.1 " " " 6.8	305	15,400	15,500
1 N HCl	305	17,400	17,700

Solvent system	Filter paper chromatography, R_F values	
	Synthetic	From kidney
n-Butanol (saturated with H_2O); NH_3 atmosphere	0.01	0.01
" diethylene glycol, HCl	0.20	0.21
" " " morpholine, H_2O; NH_3 atmosphere	0.24	0.24
n-Butanol, diethylene glycol, H_2O; NH_3 atmosphere	0.10	0.10

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$. Calculated, N 39.8; found, N 39.7

The ultraviolet absorption spectrum and filter paper chromatogram were that of 2,8-dioxyadenine. No evidence of the presence in the urine of 2,8-dioxyadenine or isoguanine could be obtained on examination by ultraviolet spectroscopy and by filter paper chromatography.

Intraperitoneal Administration of Isotopic Isoguanine—465 mg. of isoguanine sulfate hydrate (1.51 atom per cent excess N^{15} (10)) were dissolved in 4.88 ml. of 1 N NaOH and the volume was adjusted to 60 ml. by the addition of water. The solution was injected intraperitoneally once a day for 3 days into three male rats weighing a total of 872 gm. This level is equivalent to 100 mg. of adenine per kilo. The rats had received 58 ml. of the solution and were sacrificed on the 4th day. Except for the absence of ascites, the rats presented the same gross and microscopic anatomical picture as in the previous isoguanine experiment. The kidneys were processed as before and yielded 103 mg. of 2,8-dioxyadenine, which was identified spectroscopically. This amounts approximately to a 29 per cent conversion of the injected compound. The spectrum was that of 2,8-dioxyadenine.

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$. Calculated, N 39.8; found, N 40.1

Isotope analysis of the isolated compound, 1.48 atom per cent excess N^{15}, indicated direct conversion of isoguanine to 2,8-dioxyadenine with a small but significant dilution by non-isotopic precursors.

To determine the extent of conversion of the isoguanine to allantoin, the following method for the determination of allantoin based upon the isotope dilution principle (22) was developed. A total of 133 mg. of urine plus washings (under toluene) was collected during the experimental period. To 50.0 ml. of this urine were added 10.1 mg. of analytically pure allantoin[5] containing 0.602 atom per cent excess C^{13}, and after complete solution and mixing, allantoin was isolated by a procedure previously described (15).

From the C^{13} content of the isolated allantoin, 0.085 per cent excess C^{13}, it may be calculated that 160 mg. of allantoin were present in the urine collected during the course of the experiment. From this and from the N^{15} value of 0.054 atom per cent excess N^{15}, one may calculate that only 1.3 per cent of the isoguanine injected had been converted into allantoin. Thus, only 3.6 mg. of allantoin had been derived directly from the administered isoguanine.

Duplicate samples of urea isolated from the urine as the dixanthydrol derivative (15) contained 0.011 and 0.013 atom per cent excess N^{15}.

Oral Administration of Isotopic Isoguanine—Four male rats weighing

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[5] The isotopic allantoin was obtained from a biological experiment (Bendich, A., and Brown, G. B., unpublished results).

1217 gm. received during the course of 3 days 1.10 gm. of isoguanine sulfate hydrate (10), 5.40 atom per cent excess N^{15}, admixed with moistened Rockland rat diet (complete). The animals, weighing 1268 gm., were sacrificed on the 4th day, and the nucleic acids of the mixed organs were isolated and worked up as described previously (15). Various nitrogenous constituents of the urine were isolated and examined, and the results are listed in Table III.

The results show that isoguanine does not function as an effective precursor for nucleic acid purines when furnished orally to rats (16). If the assumption is made that the excretion of allantoin in this experiment was the same as in the previous experiment, it may be calculated that only about 7 per cent of the ingested isoguanine had been converted to allantoin.

TABLE III

*Feeding of Isotopic Isoguanine**

	Atom per cent excess N^{15}
Isoguanine	5.40
Nucleic acids	0.012
Purine hydrochlorides	0.007
Allantoin	0.890
Urea	0.052
Total urinary nitrogen	0.099

* 230 mg. of isoguanine per day for 3 days.

Intraperitoneal Administration of 8-Oxyadenine to Rats—Three rats totaling 603 gm. in weight received for 3 days daily intraperitoneal injections of a solution of 8-oxyadenine (11). The compound (290 mg.) was made up in 52 ml. of solution containing 2 molar equivalents of lactic acid, 5 gm. of gum acacia, 500 mg. of NaCl, and 1 drop of caprylic alcohol. In all, 196 mg. of 8-oxyadenine were injected, a level equivalent to 100 mg. of adenine per kilo per day. The animals were sacrificed on the 4th day, and the kidneys, showing typical "adenine kidney" lesions, were frozen in dry ice-alcohol, homogenized, and dried. Considerable sterile ascitic fluid had accumulated in the peritoneal cavity, but the fluid revealed no characteristic, specific absorption when examined by ultraviolet spectroscopy. The kidneys were processed as described above and 116 mg. of 2,8-dioxyadenine were obtained; this indicated a conversion of 36 per cent. For analysis, the six times recrystallized product was dried at room temperature in a vacuum desiccator over P_2O_5. The spectrum was that of 2,8-dioxyadenine.

$C_5H_5O_2N_5 \cdot \frac{1}{2}H_2O$. Calculated, N 39.8; found, N 40.1

No 8-oxyadenine or 2,8-dioxyadenine could be demonstrated in the urine by ultraviolet spectroscopy or by filter paper chromatography.

Oral Administration of 2,8-Dioxyadenine—Three rats weighing 758 gm. were given by stomach tube a total of 335 mg. of 2,8-dioxyadenine (11) suspended in 15 ml. of 10 per cent gum acacia. The animals were kept on a normal diet and were sacrificed 17 hours later. Urine and feces were collected. The kidneys appeared normal. They were extracted with 2 N HCl as described above, but the extracts yielded no 2,8-dioxyadenine and showed no specific ultraviolet absorption. No 2,8-dioxyadenine could be demonstrated in the viscera and only traces could be found in the urine. However, a hot 2 N HCl extract of the feces revealed that about 10 to 20 per cent of the dietary dioxyadenine had been eliminated presumably unabsorbed.

Intraperitoneal Administration of 2,8-Dioxyadenine—A total of 371 mg. of 2,8-dioxyadenine hemihydrate was injected into four rats intraperitoneally (weighing 757 gm.) over a period of 4 days. The compound was made up in 60 ml. of solution, containing 10 per cent gum acacia, 1 per cent NaCl, and 2 equivalents of lactic acid. The animals were sacrificed on the 5th day. There was considerable accumulation of sterile ascitic fluid in the peritoneal cavity, but no 2,8-dioxyadenine could be demonstrated in acidic extracts by ultraviolet spectroscopy. The kidneys appeared normal. When the kidneys were extracted with 2 N HCl and the extract neutralized with ammonia, no 2,8-dioxyadenine precipitated and no spectrum corresponding to this substance was obtained. It would have been possible to demonstrate the presence of 10 γ or less if it were present. Examination of 2 N HCl extracts of the organs likewise revealed no 2,8-dioxyadenine. When the urine was examined by filter paper chromatography in various media and by ultraviolet spectroscopy, no evidence for the presence of more than mere traces of 2,8-dioxyadenine could be obtained.

The authors wish to express their appreciation to Mrs. Helen Getler, Mrs. Marie Borgatta, and Mr. William D. Geren for assistance, to Mr. Roscoe C. Funk, Jr., for the microanalyses, and to Dr. Harold Beyer and Mr. John Deonarine for cooperation in the isotope determinations.

SUMMARY

The deposition of 2,8-dioxyadenine in the kidneys of the rat following the administration of adenine has been studied. The identity of the product has been confirmed. Evidence that 2,8-dioxyadenine arises as a result of a direct *in vivo* oxidation of adenine is presented. The resulting lesion has been described and has been termed "adenine kidney."

2,8-Dioxyadenine also deposits in rat kidneys when either isoguanine or 8-oxyadenine is administered, but none could be demonstrated after the administration of 2,8-dioxyadenine itself.

In connection with the biosynthesis of nucleic acids, it was found that intraperitoneally injected adenine serves as a precursor of nucleic acid adenine and guanine, but that isoguanine is inactive in this regard.

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THE COMPARATIVE AVAILABILITIES OF MIXTURES OF THE L AND DL MODIFICATIONS OF THE ESSENTIAL AMINO ACIDS FOR GROWTH IN THE RAT*

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It has heretofore frequently been assumed, though never established, that growth on an amino acid mixture may be adversely affected by the use of racemic, instead of natural, amino acids in the mixture. This was first suggested by Albanese and Irby who surmised that weight losses which they noted in young rats fed an essential amino acid mixture containing several DL-amino acids might be attributable at least in part to the "toxic effects of unnatural forms of certain amino acids that can not be utilized" (1). Their experimental results have not been confirmed. The consistency with which growth has been obtained in analogous tests before (2, 3) and since (4–7) leaves little doubt that the growth failure which they observed must have been due primarily to some other factor, probably to an isoleucine deficiency (8, 6).

Confusion has resulted from the use in most of the growth tests thus far carried out of control diets which have contained casein (4, 5, 7) or casein hydrolysates (1, 4). These have afforded better growth than have the essential amino acid mixtures. Whether the difference may be ascribed exclusively to the presence in the casein or casein hydrolysates of the unessential amino acids, which are known to accelerate growth on essential amino acid mixtures (3, 6), or may result in part from growth retardation by the DL-amino acids in the essential amino acid mixture can only be guessed.

Recently Wretlind (7) has reported that, at dietary levels of 20 to 30 per cent, mixtures of the DL modifications of the nine essential amino acids (arginine excluded) produced less rapid growth than at lower levels, hence may have been toxic. Growth retardation was also noted when only one of the DL-amino acids was greatly increased. The possibility that similar tests with L-amino acids might have produced similar results was not considered. In comparative growth tests of mixtures containing both the es-

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sential and non-essential amino acids with casein, Ramasarma, Henderson, and Elvehjem (9) noted slower growth on the former. They suggest that at the levels used "the presence of the unnatural D-isomers" in the mixtures "may not produce any symptoms of toxicity but may cause a slight depression of growth," also that "an imbalance of the amino acids in the mixtures employed" might be involved.

The purpose of this communication is to present the results of tests in which growth on mixtures of the ten essential amino acids in the DL form has been compared directly with growth on similar mixtures containing only the corresponding L modifications. So far as we are aware, such studies have not previously been reported, nor indeed have mixtures containing only the natural forms of the essential amino acids ever heretofore been employed.

EXPERIMENTAL

Most of the DL-amino acids used in these studies were made available to us by The Dow Chemical Company.[1] The DL-arginine monohydrochloride monohydrate was prepared from gelatin by the procedure of Schein and Berg (10). Part of the DL-threonine was synthesized and freed of allothreonine as outlined by West and Carter (11). The remainder was purchased from Merck and Company, as was also the DL-isoleucine.[2] Most of the DL-histidine was purchased as the monohydrochloride dihydrate from the Nutritional Biochemical Corporation. All of the DL-amino acids were recrystallized at least twice. The tryptophan, lysine monohydrochloride, histidine monohydrochloride dihydrate, and arginine monohydrochloride monohydrate were analyzed for amino nitrogen by the Van Slyke method, the methionine, phenylalanine, valine, leucine, threonine, and isoleucine for total nitrogen by Kjeldahl. In every instance the determined values agreed well with the calculated.

Table I affords data pertaining to the optical rotations of the various samples of the L-amino acids employed. It also indicates the method of resolution or isolation of the lots prepared by us and the sources of those obtained elsewhere.

Table II gives the composition of the L and the DL mixtures employed initially in the feeding tests. The L mixture afforded twice the minimal amounts of the essential amino acids tentatively indicated by Rose (23) to

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[1] We wish to thank Dr. L. S. Roehm and The Dow Chemical Company for this generosity and the cooperation extended us on several occasions.

[2] Dr. E. E. Howe of Merck and Company, Inc., has assured us that the DL-threonine is free of allothreonine, as judged by microbiological assay and solubility data, and that the DL-isoleucine is 97 per cent pure, and possibly of even higher purity.

TABLE I

Specific Rotations of L-Amino Acid Preparations Used in Feeding Tests

Amino acid	$[\alpha]_D$		Concentration per 100 ml. solution		Solvent used	Temperature of solution read	Bibliographic reference No.
	Found	Recorded in reference	As read	Recorded in reference			
	degrees	*degrees*	*gm.*	*gm.*		°*C.*	
Methionine, Lot 1	−7.3	−7.5	1.0	0.8	H_2O	25	(12)
" " 2*	−7.2		1.0		"	25	
Phenylalanine, Lot 1	−34.0	−35.14	1.0	1.9	"	20	(13)
" " 2†	−32.0		1.0		"	25	
Tryptophan, Lot 1	−32.0	−32.1	0.5	0.5	"	20	(14)
" " 2‡	−31.0		0.5		"	20	(15)
Histidine monohydrochloride monohydrate‡	+8.0	+8.0	1.0	Not recorded	3 M HCl	25	(16)
Arginine monohydrochloride§	+22.0				N HCl	25	
	+12.0	+12.3	5.0	5.0	H_2O	25	(17)
Lysine monohydrochloride, Lot 1§	+20.0		1.0		M HCl	26	
Lysine monohydrochloride, Lot 2‡	+20.1‖		1.0		" "	26	(18)
Lysine monohydrochloride, Lot 3	+21.0‖		1.0		" "	26	(19)
Valine	+29.0	+28.7	1.0	3.56	20% HCl	20	(20)
Leucine	+15.1	+15.8	1.0	4.54	20% "	20	(21)
Isoleucine	+40.0	+40.6	1.0	5.09	20% "	20	(22)
Threonine, Lot 1	−28.0	−28.3	1.0	Not recorded	H_2O	25	(11)
" " 2*	−28.0		1.0		"	25	

* We are happy to acknowledge our indebtedness to Dr. J. P. Greenstein of the National Cancer Institute who so kindly volunteered to send us a generous supply of L-methionine, and to Dr. E. E. Howe of the Research Laboratories of Merck and Company who so graciously provided us with the L-threonine needed to continue our tests.

† This lot of L-phenylalanine was purchased from the Nutritional Biochemical Corporation.

‡ L-Histidine monohydrochloride monohydrate and the samples of tryptophan and lysine monohydrochloride thus indicated were prepared in the laboratory, the first from spray-dried blood, the last two from casein by the method indicated in the reference. Application of Sullivan's naphthoquinone test indicated that the histidine monohydrochloride was free of cystine.

§ The arginine monohydrochloride and one of the samples of lysine monohydrochloride were purchased from Merck and Company.

‖ These samples were prepared through the dihydrochlorides, both of which showed specific rotations of 15.0° when 3 gm. were dissolved in water, made up to 100 cc., and polarized at 20°. The literature records 15.6° (19).

be necessary to support normal growth when the non-essential amino acids are also supplied. The DL-I mixture contained the same quantities of the L-amino acids, though supplied as components of the DL modifications. The DL-II mixture provided half as much DL-arginine, tryptophan, phenylalanine, and methionine as the DL-I mixture, and an intermediate level of DL-histidine, but the same amounts of the other five amino acids.

TABLE II

Composition of Amino Acid Mixtures

	L	DL-I	DL-II
	gm.	*gm.*	*gm.*
Valine	1.4	2.8	2.8
Leucine	1.6	3.2	3.2
Isoleucine	1.0	2.0	2.0
Lysine*	2.0	4.0	4.0
Threonine	1.0	2.0	2.0
Tryptophan	0.4	0.8	0.4
Methionine	1.2	2.4	1.2
Phenylalanine	1.4	2.8	1.4
Histidine*	0.8	1.6	1.2
Arginine*	0.4	0.8	0.4
	11.2	22.4	18.6
HCl and H_2O combined with amino acids*	0.86	1.89	1.63
$NaHCO_3$ added*	1.75	4.52	4.12
	13.81	28.81	24.35

* The L-lysine and the L- and DL-arginine were fed as the monohydrochlorides, the L-histidine as the monohydrochloride monohydrate, the DL-histidine as the monohydrochloride dihydrate, and the DL-lysine usually as the monohydrochloride. The $NaHCO_3$ was equivalent to the HCl combined with the amino acids fed. When the DL-lysine was fed as the dihydrochloride, the extra HCl and $NaHCO_3$ added 2.3 gm. to the total weights of the DL-I and DL-II mixtures.

The two isomers of tryptophan (24, 25, 6), methionine (26, 2), and phenylalanine (27) show nearly, if not quite the same, growth-promoting capacities. The indispensability of arginine becomes evident only in diets affording maximal growth (28). The D form of histidine promotes growth, but less rapidly than the L (29). On the other hand, only the L modifications of lysine (30), threonine (31), and valine, leucine, and isoleucine (2) possess appreciable growth-promoting capacities. Theoretically, therefore, the DL-II mixture should have possessed approximately the same basic growth-promoting potentialities as the L mixture.

In the first of the five series of tests conducted, each of the three amino acid mixtures was incorporated in a diet similar to that which we have heretofore commonly employed (32), except that it contained the salt mixture of Jones and Foster (33), 26 per cent of hydrogenated cottonseed oil, and dextrin instead of starch. Nicotinic acid and folic acid were included in the vitamin supplement, in addition to the factors noted in the reference cited. Subsequent trial of the low fat type of diet employed by Rose, Oesterling, and Womack (6) proved it to be so superior that its use was adopted. Hence, in all of the other tests, each 100 gm. of diet contained, in addition to the amino acid mixture (or other source of protein nitrogen), corn oil 2.0, vitamin A and D concentrate[3] 0.08, salt mixture (33) 4.0, Cellu flour 2.0, inositol 0.1, choline chloride 0.2, liver extract 0.4,[4] vitamin B_{12} concentrate[5] 0.1 gm., and dextrin to make 100 gm. To each kilo of ration were added thiamine hydrochloride 5.0, riboflavin 10.0, pyridoxine hydrochloride 5.0, nicotinic acid 5.0, calcium pantothenate 25.0, *p*-aminobenzoic acid 300.0, α-tocopherol 25.0, 2-methyl-1,4-naphthoquinone 2.0, biotin 0.1, and folic acid 0.1 mg.

Weanling rats of the Sprague-Dawley strain were used throughout. They were housed individually and allowed continual access to food and water. The test periods were 28 days long.

Table III shows the comparative growth responses obtained on the high fat and low fat types of diet. In the animals fed the high fat diet (Series I), growth on the DL-II mixture exceeded that on the L mixture. In the rats fed the low fat diet (Series II), growth on these mixtures was approximately the same, though inferior to that on a casein hydrolysate.[6] Growth on the DL-I mixture was definitely inferior in both series to that on either the L or the DL-II mixture. No gross abnormality was detectable on postmortem examination of the animals fed the L and the DL-II mixtures, but all of the animals fed the DL-I mixtures showed darkened spleens.

Table IV summarizes data which indicate (Series III) that the agent chiefly responsible for the growth retardation on the DL-I mixture was the extra DL-methionine which it contained. In contrast with the marked

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[3] Oleum percomorphum, marketed by Mead Johnson and Company. Per gm. it contained 60,000 U. S. P. units of vitamin A and 8500 U. S. P. units of vitamin D.

[4] Liver concentrate 1:20, kindly supplied by The Wilson Laboratories, through the courtesy of Dr. S. W. Hier.

[5] We are indebted to Dr. L. R. Hines and E. R. Squibb and Sons for a generous supply of Preparation ER-72-4, which contained 15 γ of vitamin B_{12} per gm., with starch as the diluent.

[6] This was an acid hydrolysate made available to us by The Upjohn Company, through the courtesy of Dr. John T. Correll and Dr. Curtis E. Meyer. It was fortified as indicated in Table III.

TABLE III

Growth in 28 Days on Diets Containing Essential Amino Acid Mixtures Composed Entirely of L- or Entirely of DL-Amino Acids

Series No.	No. of rats	Basal diet*	Gain in weight	Food consumed
			gm.	*gm.*
I	3♂, 3♀	L	33 ± 11	101 ± 11
	2♂, 3♀	DL-I	28 ± 6	87 ± 11
	3♂, 3♀	DL-II	49 ± 11	118 ± 20
II	2♂, 2♀	L	80 ± 10	244 ± 26
	5♂, 5♀	DL-I	44 ± 12	137 ± 27
	7♂, 7♀	DL-II	76 ± 8	217 ± 19
	3♂, 3♀	CH-11.7	99 ± 9	288 ± 32

* The basal diet used in Series I was high in fat, that in Series II was low. L indicates 11.2 per cent of L-amino acids, DL-I 22.4 per cent of DL-amino acids, and DL-II 18.6 per cent of DL-amino acids. CH-11.7 indicates a diet which contained 11.7 per cent of an acid hydrolysate of casein, instead of a mixture of purified amino acids. The hydrolysate was fortified with 0.6 gm. of DL-methionine and 1.1 gm. of DL-tryptophan per 100 gm., and was isonitrogenous with the L-amino acid mixture.

TABLE IV

Growth Retardation Induced in 28 Days by Supplementing DL Mixtures of Essential Amino Acids with Additional DL-, L-, or D-Methionine

Series No.	No. of rats	Basal diet	Supplement per 100 gm. diet	Gains in weight	Food consumption
			gm.	*gm.*	*gm.*
III	1♂, 4♀	DL-II	None	70 ± 8	208 ± 20
	2♂, 3♀	"	DL-Histidine 0.4	68 ± 4	195 ± 11
	3♂, 2♀	"	DL-Tryptophan 0.4	65 ± 9	214 ± 26
	3♂, 2♀	"	DL-Arginine 0.4	73 ± 3	211 ± 13
	3♂, 2♀	"	DL-Phenylalanine 1.4	73 ± 11	219 ± 15
	2♂, 3♀	"	DL-Methionine 1.2	41 ± 11	152 ± 10
	2♀	"	All of above*	40 ± 6	146 ± 6
IV	5♂	"	None	80 ± 4	233 ± 5
	5♂	"	DL-Methionine 1.2	68 ± 8	185 ± 9
	5♂	"	L-Methionine 1.2	48 ± 6	144 ± 14
	5♂	"	D-Methionine 1.2	71 ± 13	189 ± 14
	5♂	"	DL-Histidine 0.4, arginine 0.4, tryptophan 0.4, and phenylalanine 1.4	100 ± 10	268 ± 10
	5♂	"	Same + DL-methionine 1.2*	56 ± 6	168 ± 10
	5♂	CH-11.7†	None	92 ± 12	275 ± 19
	5♂	"	DL-Methionine 0.6	81 ± 15	236 ± 50
	5♂	"	" 1.8	40 ± 6	144 ± 24
	5♂	"	" 3.0	13 ± 7	109 ± 11

* The supplements indicated converted the DL-II into the DL-I mixture.

† Per 100 gm., this diet contained 11.7 gm. of an acid hydrolysate of casein which had been fortified as indicated in Table III.

growth retardation induced upon increasing the DL-methionine to the level fed in the DL-I diet, variations in growth induced by increasing the other amino acids individually to the levels prevailing in the DL-I mixture were minor and of doubtful significance.

The data in Series IV show that the simultaneous addition to the DL-II mixture of 0.4 gm. each of DL-histidine, tryptophan, and arginine and 1.4 gm. of phenylalanine increased the rate of growth significantly. They also indicate that the growth retardation induced by the L isomer of methionine was significantly greater than that induced by either the DL or the D form. Supplementary tests with casein hydrolysate showed that as methionine was added in increasing quantities the capacity of the diet to promote growth was progressively impaired. Darkened spleens were found only in the animals fed the DL-II mixtures supplemented with methionine and in the animals fed diets containing the hydrolysate to which 1.8 gm. or more of methionine had been added per 100 gm. of diet.

TABLE V

Series V, Growth in 28 Days on L and DL Mixtures of Essential Amino Acids Made Isonitrogenous by Addition of Glycine and Ammonium Citrate

No. of rats	Basal diet	Supplement per 100 gm. diet	Gains in weight	Food consumption
		gm.	*gm.*	*gm.*
3♂	L-a[*]	None	97 ± 3	263 ± 16
3♂	"	Glycine 3.7 and NH_4 citrate 2.9[†]	97 ± 11	254 ± 36
5♂	DL-II-a[*]	None	97 ± 11	247 ± 25
4♂	"	Glycine 3.7 and NH_4 citrate 2.9	93 ± 15	258 ± 26
5♂	"	DL-Phenylalanine 0.8, histidine 0.4, tryptophan 0.4, and arginine 0.4[‡]	100 ± 16	270 ± 30
4♂	CH-12.5[§]	None	106 ± 9	310 ± 19
5♂	"	DL-Methionine 2.0	63 ± 8	200 ± 22
5♂	"	" 2.0, glycine 2.0, and L-arginine 1.0	97 ± 19	265 ± 39
3♂	CH-20.6[§]	None	159 ± 4	362 ± 18
3♂	CH-28.7[§]	"	158 ± 4	381 ± 13

[*] The L-a diet contained 0.6 gm. more L-phenylalanine per 100 gm. than the L diet, the DL-II-a diet 0.6 gm. more DL-phenylalanine than the DL-II.

[†] These supplements compensated for the extra nitrogen supplied in the DL-II-a mixture.

[‡] The DL-II-a diet plus these supplements was identical to the DL-II diet plus the supplements of these four amino acids used in Series IV (Table IV).

[§] The hydrolysate was from the lot used also in Series II and Series IV. At the 12.5 per cent level, the diet containing it was isonitrogenous with the L-a diet, at the 20.6 per cent level with the DL-II-a diet, and at the 28.7 per cent level with the DL-II-a diet which had been supplemented with the glycine and the ammonium citrate.

In Series V (Table V), the phenylalanine content of each of the amino acid mixtures used was increased by 0.6 gm. per 100 gm. of diet. Critical examination of the rates of growth on the L-a and the DL-II-a diets reveals little or no difference. The extra nitrogen supplied some of the animals as glycine and ammonium citrate neither enhanced nor significantly retarded the growth rate.

Gains on the L-a and the DL-II-a mixtures approximated those on the diet which contained 12.5 per cent of the casein hydrolysate, but it should be noted that at that level the hydrolysate may not have provided enough tryptophan for optimal growth response. Gains on the diet containing 12.5 per cent of the hydrolysate were considerably less than those on diets containing 20.6 and 28.7 per cent. Incidentally, growth on the latter equaled that attained on a stock diet of Purina laboratory chow.

Unrecorded paired feeding tests indicated that restriction of the food intake was probably largely responsible for the slower growth of the animals receiving the 2 per cent supplement of DL-methionine, but not for the darkened spleens. Supplementation of the high methionine type of diet with glycine and L-arginine stimulated food consumption and growth, but did not quite prevent the spleen darkening. Preliminary tests have indicated that the addition of 1 and 2 per cent of choline to the CH-12.5 diet produces darkening of the spleen, but does not promote marked growth retardation.

DISCUSSION

The data obtained seem to us to prove rather conclusively that in our tests the D components of the DL mixtures exerted no appreciable deleterious effect *per se*, despite the fact that they were present in amounts ranging from 9.3 to 10.6 per cent of the various diets.

The growth-retarding effect in the rat of relatively high levels of DL-methionine has been previously noted. Kade and Sheperd (34) observed that the addition of 1 per cent of DL-methionine afforded increased utilization of an 8 per cent casein diet, that 1.5 per cent promoted no enhancement, and that 2 to 3 per cent was inhibitory. Brown and Allison have reported marked weight losses on diets containing 12 per cent of casein and 4.8 per cent of DL-methionine (35). In tests of the DL forms of the essential amino acids as dietary supplements, Wretlind (7) has noted that DL-methionine can be increased the least of all without causing retarded growth. Howe *et al.* (36) have found it the most poorly tolerated of the racemic amino acids upon intravenous infusion into dogs.

The observation (Series IV, Table IV) that the growth retardation induced by increasing the D-methionine content of the diet was appreciably less than that caused by adding the natural L isomer is of particular interest. It is inconsistent with the supposition that the D forms of the essential amino acids are the more likely to cause "toxic effects" or depression of growth (1, 7, 9), especially if they are unable to promote growth (1). Smaller growth retardation by D-methionine may result from its more ready escape into the urine.

Histological examinations were made of the spleens of the animals on the various diets, with use of hematoxylin-eosin and iron stains. The kidneys, livers, lymph nodes, and bone marrow of about half of each group were also examined. We are greatly indebted to Dr. J. R. Carter of the Department of Pathology for making these studies. He reports finding no significant abnormalities in any organ except the spleen. Detailed cellular counts of the bone marrow were not made, but no striking abnormalities were detected. In all of the animals fed the higher levels of DL-, D-, or L-methionine, the spleen showed varying degrees of congestion, with formation of hemosiderin pigment. The normal architectural pattern was well preserved, and no significant cellular changes were observed. No histological differences were noted between animals fed the L- and those fed the DL-amino acid mixtures with the lower level of methionine. Hemosiderin was not found in their spleens in significant amounts.

The reason for the findings on the spleens of the rats fed the higher levels of methionine is uncertain. Examinations of the cellular components of the blood have thus far afforded no suggestive clues beyond a low leucocyte count. Reticulocyte and erythrocyte counts, hemoglobin (Newcomer), and fragility tests have shown no marked divergencies.[7] The fact that spleen darkening is induced also by feeding excess choline seems to indicate that labile methyl groups may be at least partially responsible. Possibly alleviation of the condition upon the addition of glycine and arginine to the diets containing excess methionine (Table V) may reflect the conversion of such groups to creatine. In studies made in the chick, McKittrick (37) noted growth depression upon feeding excess methionine, but none when choline chloride was fed, unless the diet was supplemented with homocystine. Counteraction of the growth-depressing effect was observed upon adding glycocyamine or serine. In the rat, Brown and Allison (35) noted less growth retardation and an increased creatinine excretion when L-arginine was added to their high methionine diets. The growth retardation noted in our studies was not altered by doubling the vitamin allotment (vitamin B_{12} excepted).

Rose has reported growth enhancement upon supplementing diets containing 5.8 and 2.8 per cent of only the essential amino acids with glycine and ammonium citrate (38). It seems probable that our diets may already have provided as much extra nitrogen as the organism could use for the synthesis of unfurnished amino acids. Some justification for assuming that the extra nitrogen might have stimulated growth, had our mixtures been fed at lower levels, is found in the better growth in Series I (Table III) of the rats fed the DL-II mixture than in those receiving only the L-amino acids. In Series I the food consumption, hence the amino acid intake, was much smaller than in the subsequent tests. Little information is available concerning the minimal or optimal proportions of the essential amino acids for promoting growth, when the essential amino acids constitute the sole source of protein nitrogen. We have some evidence that our original DL-II and L mixtures may have contained more threonine than necessary. On the other hand, they may have contained too little phenylalanine and possibly border line amounts of some of the other amino acids.

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[7] The cooperation of Mr. Wm. A. Phillips who made the blood tests is appreciated.

SUMMARY

Rats fed the L forms of the ten essential amino acids as components of a DL mixture constituting 22.4 per cent of the diet grew less well than control rats fed only the L isomers at a dietary level of 11.2 per cent. When allowance was made for the growth-promoting capacities of the D components of the DL mixture, and only half as much DL-phenylalanine, tryptophan, methionine, and arginine and an intermediate level of DL-histidine were included, the resulting 18.6 per cent of DL-amino acids promoted as good growth as that attained on the L mixture.

By adding each of the lowered amino acids in turn to the 18.6 per cent DL diet, the growth retardation was traced to excess methionine. Removal of half of the DL-methionine completely overcame the growth-retarding effect of the 22.4 per cent DL mixture. Comparative tests showed that the growth retardation produced by the natural L isomer of methionine was greater than that produced by either the DL or the D modification.

No differences were noted in comparative tests of the blood or in postmortem examination of the rats fed the L and DL mixtures which did not contain excessive amounts of methionine. On the other hand, in the rats fed excessive amounts of methionine, either in amino acid mixtures or as supplements in diets containing casein or casein hydrolysates, the spleens were dark and were congested with hemosiderin pigment. Additions of glycine and arginine overcame the growth retardation, but not entirely the spleen congestion. Diets containing casein hydrolysates to which 1 and 2 per cent of choline were added produced darkened and congested spleens, but did not retard the growth. An extension of these tests is planned to determine to what extent labile methyl groups may be involved.

Increasing the phenylalanine contents of the 11.2 per cent L- and the 18.6

per cent DL-amino acid diets by 0.6 per cent seemed to improve the growth promotion by each to about the same extent. Addition to these diets of extra nitrogen in the forms of glycine and ammonium citrate did not further enhance, nor did it retard, the growth. Possible reasons are suggested.

It seems fair to conclude that, contrary to the often repeated conjecture that the D forms of the essential amino acids may be toxic in the rat, proportionately large amounts can actually be fed as components of DL-amino acid mixtures without producing any apparent growth-retarding or other deleterious effect. Moreover, when fed in excessive amounts, the L form of an amino acid may, as has been shown in the case of methionine, be even more deleterious than its D modification.

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THE EFFECT OF PREVIOUS CARBOHYDRATE DEPRIVATION ON THE SYNTHESIS OF ALANINE BY LIVER IN VITRO*

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While studying the influence of the hormones on alanine synthesis by the liver, we observed that occasionally our normal control animal gave an unusually low value, which seemed to be associated with a low intake of food the previous day. That the composition of the diet might affect the activity of a tissue has been repeatedly observed. Miller (1), Potter and Klug (2), Kaplan and Greenberg (3), and Seifter, Harkness, Feldman, Rubin, and Muntwyler (4) all have presented evidence that fasting or the administration of modified diets for long periods affected the enzymatic activity of tissue. Deamination of alanine by mouse liver slices was found by Kaplanskii, Berezovskaya, and Shmerling (5) to be completely stopped when the animals were subjected for 20 to 35 days to a diet low in protein. Neber (6) observed that liver slices of guinea pigs fasted for 2 days produced less amino nitrogen from pyruvic acid and ammonium carbonate. In view of the many mechanisms that might be involved, we decided to study the effect of fasting and of variation in composition of the diet on the synthesis of alanine by rat liver slices.

Methods

Adult rats of the Wistar strain were given the following basic diet for five days: casein 25 per cent, sucrose 50 per cent, Crisco 20 per cent, salt mix 5 per cent, and a vitamin supplement of Vi-Penta drops (La Roche) 0.5 ml. per kilo of diet. After this period, the diet was altered either by fasting or by changing its composition with respect to the percentage of protein, fat, or carbohydrate.

Observations were made on surviving liver slices prepared as for the Warburg technique. The animals were killed by a blow and the liver was immediately excised and sliced into 20 ml. of a bicarbonate buffered Krebs' solution at pH 7.2 containing phosphate (7). In control experiments, about 100 mg. of liver slices (dry weight) were transferred to a 250 ml. Erlenmeyer flask containing 20 ml. of the above solution, or, in test experiments, to a flask with the same solution containing 0.1 M sodium pyruvate and 0.04 M ammonium carbonate. The tissues were then shaken

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in an atmosphere of 5 per cent CO_2 and 95 per cent O_2 for 2 hours at 37.5°. At the end of this period, they were removed by filtration, washed, dried at 104° overnight, and weighed. After having been made alkaline to phenolphthalein and boiled for the removal of ammonia (checked by nesslerization), the medium was analyzed for the increase in amino nitrogen by the nitrous acid method of Van Slyke (8).

Kritzmann (9) and Wiss (10) have demonstrated the formation of alanine from pyruvic acid and ammonia by liver slices and homogenate under experimental conditions essentially similar to ours. Experiments of our own with chromatography have shown that the increase in amino nitrogen is due almost entirely to formation of alanine. Traces of aspartic or glutamic acid are also formed. Since no amino nitrogen was found when pyruvate and ammonium carbonate were incubated in the medium without tissue, it was assumed that the small amount of amino nitrogen appearing in the flasks containing tissue and medium without substrates represented that which was simply leached from the tissue. This was deducted from the quantity found in the test flasks, and the difference represented the synthesized amino nitrogen. The results are given in terms of micrograms of amino nitrogen per mg. of dry weight of tissue per hour.

Results

The effect of fasting on the ability of the rat liver to synthesize alanine is shown in Fig. 1. The average amount of amino nitrogen formed by livers of twenty-two fed control animals is 7.1 ± 0.26 γ per mg. of dry tissue per hour. Compared with this value, a profound depression in the formation of alanine following periods of fasting greater than 12 hours is observed. In ten experiments each, 24 and 48 hour fasts gave values of 1.2 ± 0.15 γ per mg. and 1.1 ± 0.14 γ per mg. respectively, while those for 72 and 96 hours were somewhat higher, but greatly below normal, being 2.6 ± 0.65 and 2.7 ± 1.04 respectively. In all cases, this deficiency of alanine synthesis produced by fasting could speedily be restored to normal by feeding the animals the standard diet. Thus, the synthesis amounted to 7.4 γ per mg. of tissue per hour when the standard diet was given for 24 hours after a 24 hour fast, and 6.2 γ per mg. when it was given after a 72 hour fast.

In order to ascertain whether this failure of synthesis after fasting was due to a deficiency of protein, carbohydrate, or fat, other groups of animals that had been placed on diets with varying amounts of these constituents were studied. Table I shows the results obtained when the animals had been fed these modified diets for 24 hours. The percentage composition in terms of total calories of each test diet, as well as the tota

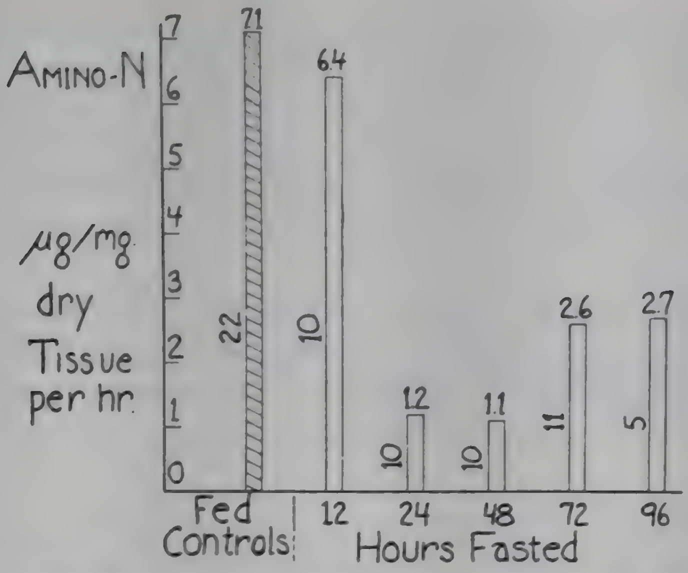

FIG. 1. Amino nitrogen synthesized by liver slices from rats after various periods of fasting. The numbers along the sides of the bars indicate the number of experiments, while those at the top of the bars are the average values obtained for each group of experiments in micrograms per mg.

TABLE I

Effect of Diet on Alanine Synthesis by Rat Liver

Amino nitrogen synthesized by liver slices from rats placed for 24 hours on the modified diets.

No. of experiments	Composition of diet			Amount eaten	Calories eaten per 24 hrs.	Available carbohydrate	Amino nitrogen
	Carbohydrate	Fat	Protein				
	per cent calories	*per cent calories*	*per cent calories*	*gm. per 24 hrs.*		*gm. per 24 hrs.*	*γ per mg. dry tissue per hr.*
10	0	0	0	0	0	0	1.2 ± 0.15*
1	0	100	0	13	117	1.3	1.8
8	0	80	20	9	61	2.0	3.3 ± 0.69
8	0	38	62	8	38	3.2	3.8 ± 0.41
8	0	0	100	9.7	39	4.9	4.4 ± 0.59
8	47	53	0	11	59	7.4	6.2 ± 0.35
22	42 (Basic diet)	37	21	13	64	8.7	7.1 ± 0.26
3	62	38	0	12	58	9.2	8.1 ± 1.02
4	78	0	22	13	52	11.0	8.2 ± 0.33

* Deviation measure = standard deviation of the mean.

available carbohydrate, calculated as 10 per cent of the fat, 50 per cent of the protein, and 100 per cent of the carbohydrate eaten, is shown in Table I. In some cases, animals were kept on these diets for 48 and 72 hours, but, since the values obtained were not significantly different from those resulting after 24 hours, they were not included.

It appears from Table I that the deficiency in alanine synthesis by these livers is a function of the total available carbohydrate in the diet. Plotting the available carbohydrate against the micrograms of amino nitrogen produced by these tissues (Fig. 2) shows this relationship to be a linear one.

In two experiments, the addition of glucose to the medium in a concentration of 50 mg. per cent had no effect on the diminished synthesis of alanine by the liver from the fasted rat. An average value of 1.38 γ of alanine per mg. of dry tissue was obtained in these two experiments.

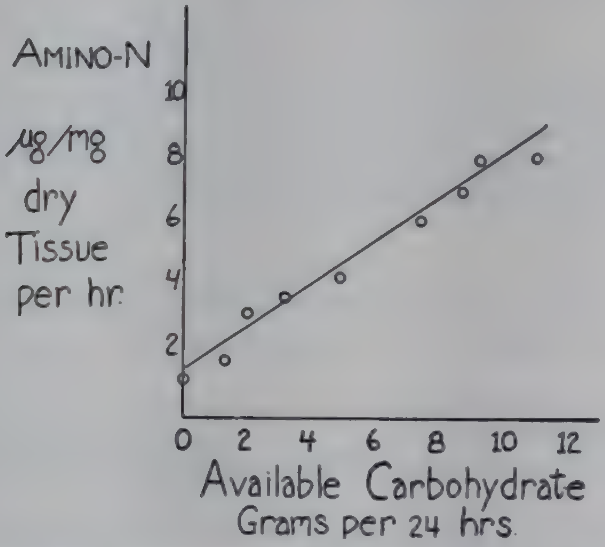

FIG. 2. Amino nitrogen synthesized by liver slices from rats placed for 24 hours on modified diets. Ordinate, micrograms of amino nitrogen per mg. of dry liver tissue per hour; abscissa, total amount of carbohydrate in gm. derivable from the diet eaten per 24 hours.

DISCUSSION

The most striking feature of these experiments is the rapidity and severity with which a dietary deficiency can affect a tissue function. Even in so short a period as 24 hours, fasting can almost completely abolish a synthetic process exhibited by the liver. The results indicate that the failure in alanine synthesis in these tissues is due to carbohydrate deficiency specifically, and not to a lack of non-specific sources of energy. This appears to be an indirect effect since addition of glucose *in vitro* did not increase the synthesis. A reductive amination would involve the need for a hydrogen donor, and it is not unreasonable to postulate that the deficiency, as observed in our experiments, was due to the lack of such a donor. Although we confirm Wiss' (10) observation that the synthesis does not occur anaerobically, it is unlikely under our conditions of pyruvate excess that there should be a failure of carbohydrate oxidation, and hence a hydrogen donor deficiency below the pyruvate level. In searching for a possible place where such a deficiency might occur, we should take into account the observation of Kaplan and Greenberg (3) that a reduced content of adenosine triphosphate is found in the livers of carbohydrate-starved animals. This would favor a hydrogen donor deficiency at the triose stage of carbohydrate metabolism, and may explain the failure for the synthesis of alanine under the conditions of our experiments.

SUMMARY

Liver slices from fasted rats or from animals on diets deficient in carbohydrate show a marked reduction in the ability to synthesize alanine from pyruvic acid and ammonia.

The deficiency in alanine synthesis seems to be a direct function of the total available carbohydrate in the diet.

The addition of glucose to the medium does not increase the synthesis of alanine by the livers of fasted rats.

The possible mechanisms involved in this failure are discussed.

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THE SPECTROPHOTOMETRIC DETERMINATION OF CARBON MONOXIDE

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The spectrophotometry of carboxyhemoglobin and oxyhemoglobin has been studied by Cherbuliez (1), Hüfner (2), Newcomer (3), Haurowitz (4), Kennedy (5), and Warburg (6). The absorption curves of these pigments (taken from a compilation of their results) are shown in Fig. 1.

In 1900 Hüfner (7) worked out a spectrophotometric method for the determination of carboxyhemoglobin in the presence of oxyhemoglobin. He utilized the principle that, in mixtures of two pigments, if the quotient D_1/D_2 (in which D_1 equals the optical density at wave-length I and D_2 equals the optical density at wave-length II) of one pigment is A, and the quotient D_1/D_2 for the other pigment is B, then any mixture of the two pigments will have a D_1/D_2 value somewhere between A and B, depending on the concentration of the pigments. His mathematical treatment is similar to that presented by Gibb (8).

Hüfner chose the wave-lengths 541 and 560 mμ and showed that D_{541}/D_{560} for pure carboxyhemoglobin is 1.10 and for pure oxyhemoglobin 1.58, and that mixtures of the two have D_1/D_2 values between these two figures. Heilmeyer (9) substituted the quotient D_{576}/D_{560} which is 0.88 for carboxyhemoglobin and is 1.72 for oxyhemoglobin. Mixtures of the two have values between these two limits. The difference between the quotient for pure carboxyhemoglobin and the quotient for pure oxyhemoglobin in this case is more substantial than the difference obtained with Hüfner's quotient. With both of these quotients, however, the difference in the values obtained with pure pigments is still quite small and requires extremely accurate technique and instruments.

We felt that a more satisfactory quotient could be obtained by measurement of the optical density of carboxyhemoglobin in the presence of reduced hemoglobin. Oxyhemoglobin is completely reduced in the presence of small amounts of sodium hydrosulfite, whereas carboxyhemoglobin is not affected. The curves for reduced oxyhemoglobin and "reduced" carboxyhemoglobin are shown in Fig. 2. From a consideration of these curves, the wave-lengths 555 and 480 mμ have been chosen as most suitable for the determination of carboxyhemoglobin in the mixture.

Procedure

1 ml. of oxalated blood is diluted to 100 ml. with 0.4 per cent ammonia. 3 ml. are placed in a cuvette and about 10 mg. of pure sodium hydrosulfite are added. The solution is mixed by inverting ten times and the densities at 555 and 480 mμ are read at once against 0.4 per cent ammonia as a blank. The value of D_{555}/D_{480} is calculated and the percentage of carboxyhemoglobin is read from the prepared concentration-quotient curve. All read-

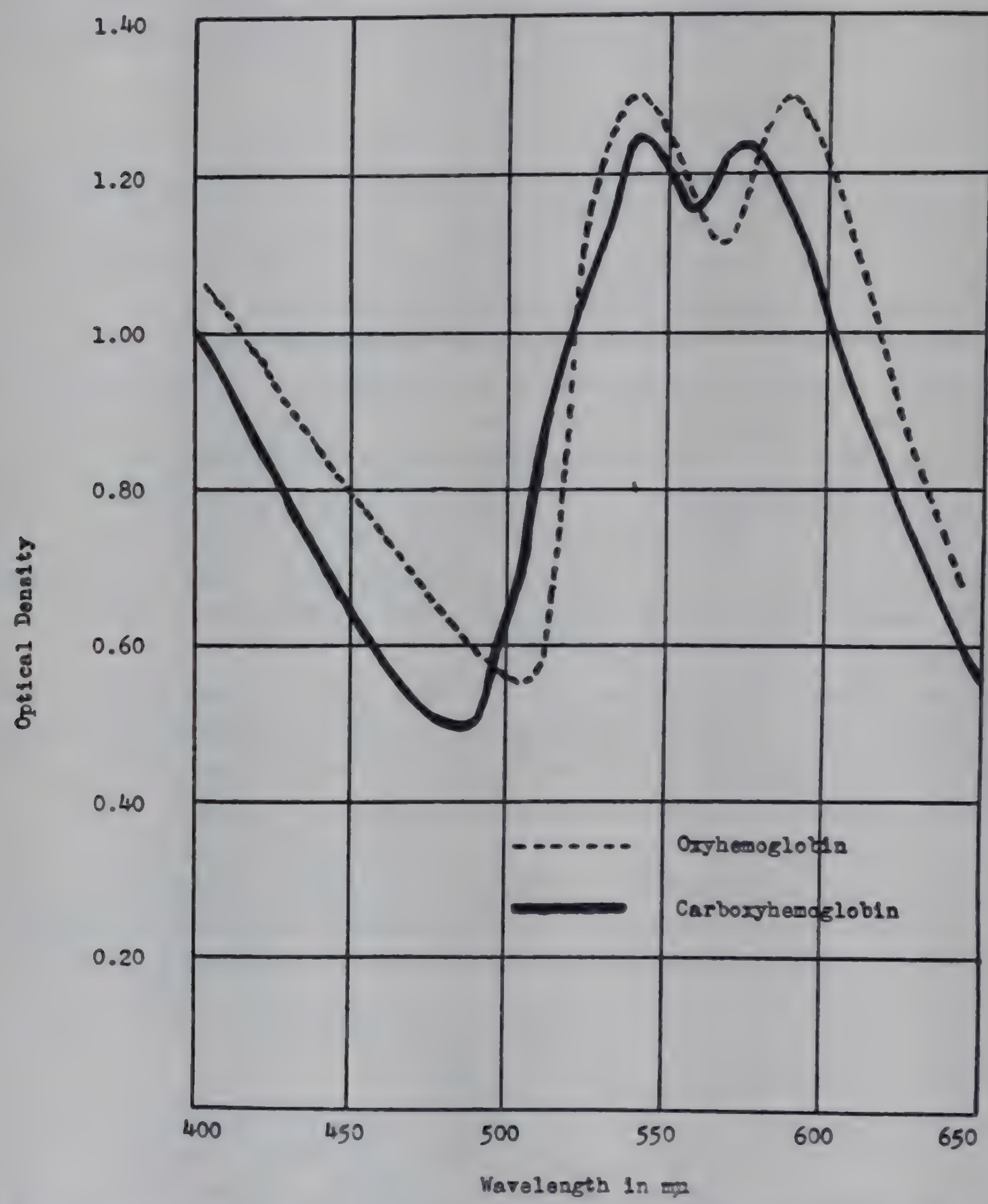

FIG. 1. Absorption curves of oxyhemoglobin and carboxyhemoglobin (1:100 dilution in 0.4 per cent ammonia).

ings were made on the Beckman model DU quartz spectrophotometer in 1 cm. quartz cells.

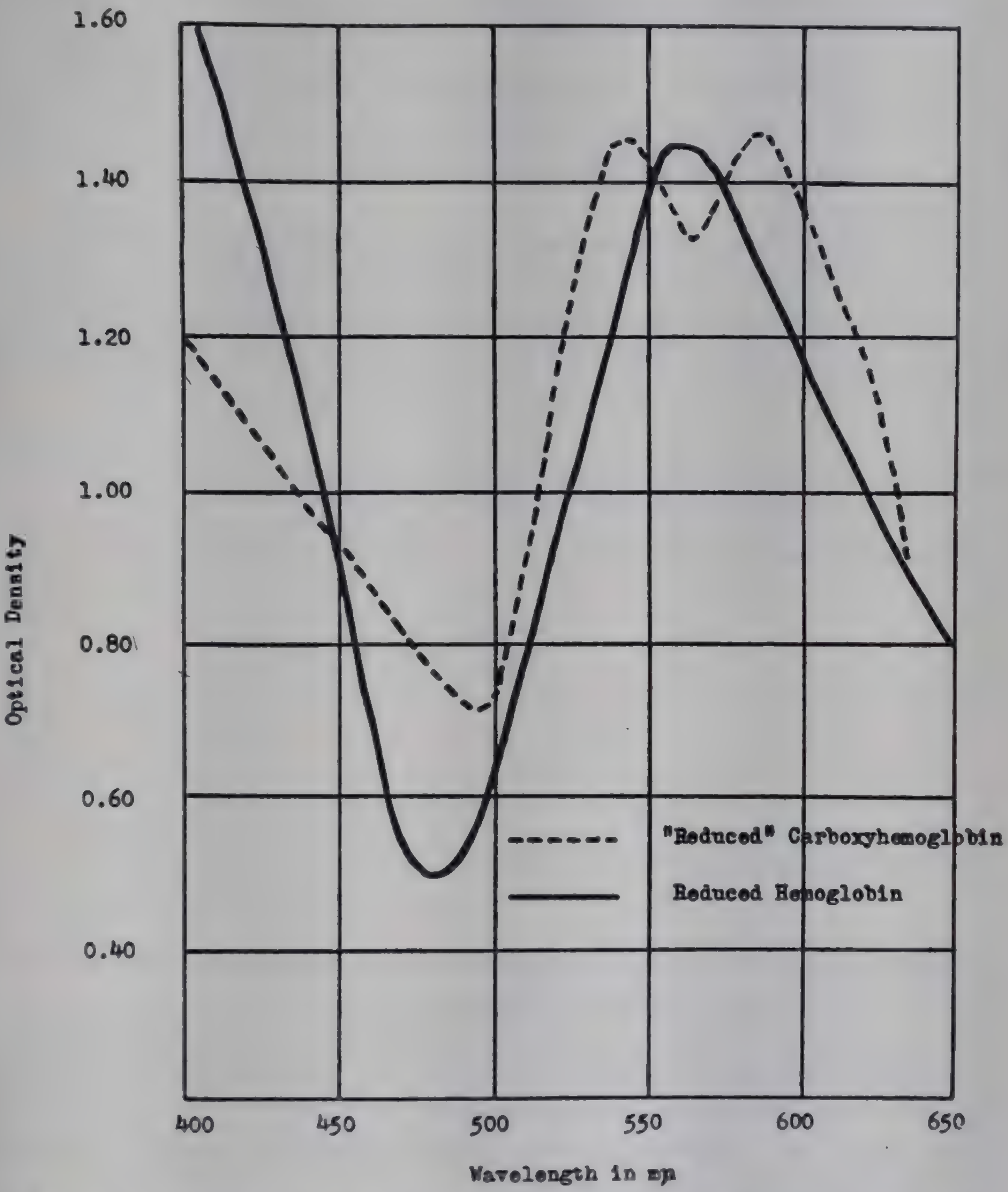

FIG. 2. Absorption curves of reduced hemoglobin and "reduced" carboxyhemoglobin (1:100 dilution in 0.4 per cent ammonia).

Determination of Quotient D_{555}/D_{480} for Carboxyhemoglobin and Reduced Hemoglobin—Twenty samples of oxalated blood from hospital patients known not to have been exposed to carbon monoxide were obtained. 5 ml. of the sample were placed in a 250 ml. separatory funnel, and the air remaining was displaced with pure oxygen. The funnel was capped and rotated for ½ hour to saturate the hemoglobin with oxygen. A second 5 ml. sample of the same blood was treated similarly, with carbon monoxide to displace the air in the separatory funnel. In this manner, samples of oxyhemoglobin and carboxyhemoglobin were prepared. 1 ml. portions of each were diluted to 100 ml. with 0.4 per cent ammonia and analyzed as outlined in the "Procedure." The quotient D_{555}/D_{480} for reduced oxyhemoglobin is 3.15 ± 0.05, and for "reduced" carboxyhemoglobin 1.94 ± 0.05. These figures agree closely with quotients calculated from data presented by Heilmeyer (10).

Preparation of Concentration-Quotient Curve—Mixtures of blood containing varying amounts of carbon monoxide were prepared and analyzed for carbon monoxide by the Van Slyke volumetric method (11). These samples were then diluted 1:100 with 0.4 per cent ammonia and analyzed according to the procedure outlined above. The results are shown in Table I. The concentration-quotient curve is shown in Fig. 3.

TABLE I

Relationship between Per Cent Carbon Monoxide and Quotient D_{555}/D_{480} for Reduced Hemoglobin and "Reduced" Carboxyhemoglobin

Per cent carbon monoxide by Van Slyke analysis	Quotient D_{555}/D_{480}
0	3.15
10	2.97
20	2.81
30	2.67
40	2.54
50	2.41
60	2.31
80	2.12
100	1.94

As a final check on the accuracy of the method, a series of bloods taken from patients exposed to carbon monoxide was analyzed by both the Van Slyke method and the spectrophotometric method, by the use of Heilmeyer's quotient, Hüfner's quotient, and the reduced quotient D_{555}/D_{480}. These results are shown in Table II.

From a consideration of these figures, it can be seen that for values of carbon monoxide saturation below 20 per cent the three spectrophotometric methods give results closely comparable with those by the Van Slyke gasometric method. However, with values above 20 per cent, and especially in the range 50 to 80 per cent, only the reduced quotient D_{555}/D_{480} gives results which agree closely with the Van Slyke procedure.

The presence of other pigments introduces errors which invalidate the two pigment calculation (8). However, methemoglobin does not interfere since it is reduced with sodium hydrosulfite ((10) p. 105). That methemoglobin does not interfere was shown by analysis of several blood samples containing both methemoglobin and carboxyhemoglobin. These results are shown in Table III. The percentage of methemoglobin was determined by Heilmeyer's method ((10) pp. 103–104).

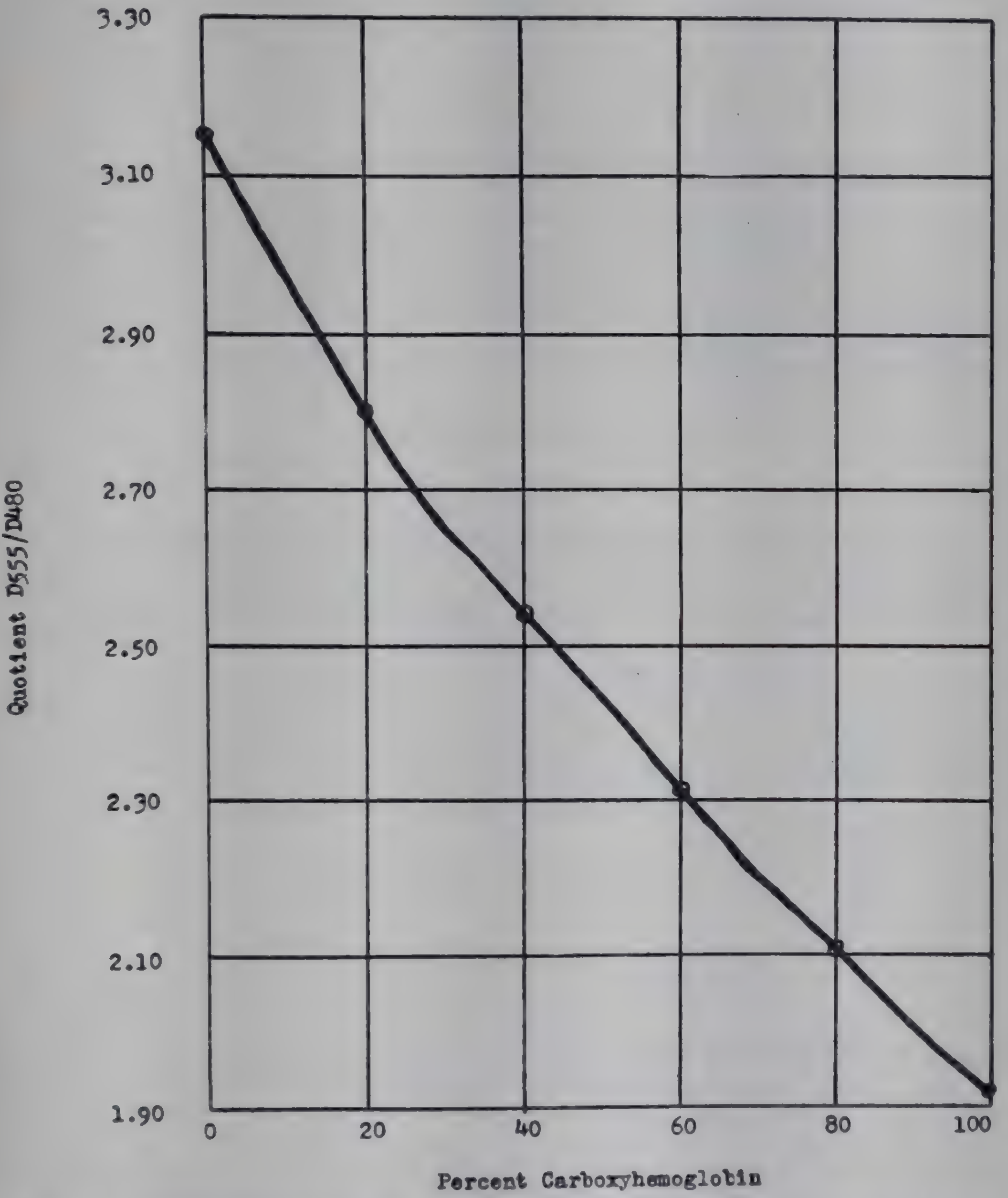

FIG. 3. Concentration-quotient curve. Proportion of carboxyhemoglobin in a mixture of oxyhemoglobin and carboxyhemoglobin determined by means of the reduced quotient D_{555}/D_{480}.

TABLE II

Comparison of Results for Carbon Monoxide Content of Blood by Various Methods

Case No.	Per cent carbon monoxide saturation			
	Van Slyke gasometric method	Hüfner's method, D_{541}/D_{560}	Heilmeyer's method, D_{576}/D_{560}	Reduced method, D_{555}/D_{480}
1	74.1	55.2	51.4	73.2
2	82.0	68.0	64.0	81.5
3	73.5	44.0	43.0	72.5
4	18.7	13.0	19.0	19.0
5	77.8	59.0	54.0	78.5
6	10.7	9.5	9.5	10.0
7	14.5	14.5	13.9	14.0
8	83.0	69.0	72.0	82.0
9	39.6	27.0	30.0	40.0
10	71.5	60.0	55.0	72.0
11	87.0	70.0	67.0	86.0
12	31.5	23.0	22.0	30.0
13	43.5	37.0	33.0	41.0
14	29.0	20.0	19.0	30.0

TABLE III

Determination of Carbon Monoxide in Presence of Methemoglobin

Per cent methemoglobin	Per cent carbon monoxide	
	Van Slyke method	Quotient D_{555}/D_{480}
7.5	0	0
17.2	1.5	2.0
9.3	10.9	9.9
72	0	0
9.0	2.5	3.2
26	0	0
16	31	32
47	0	0

TABLE IV

Stability of Reduced Quotient D_{555}/D_{480} of Unhemolyzed Blood in Determination of Carbon Monoxide

Time on ice	Per cent carbon monoxide	
	Van Slyke method	Quotient method, D_{555}/D_{480}
hrs.		
0	82.0	81.0
4	82.2	81.0
8	82.0	81.5
16	82.4	81.0
24	82.2	81.4

Blood which is hemolyzed, or which has been standing for several days, as is the usual case with post mortem samples, contains other pigments arising from the breakdown of hemoglobin which make it unsuitable for analysis by this method. If precautions are taken to avoid hemolysis, the reduced quotient of refrigerated blood remains constant for several days. This is shown in Table IV.

SUMMARY

A method for the spectrophotometric determination of carbon monoxide in blood has been presented. The method utilizes the quotient D_{555}/D_{480} in the two pigment solution consisting of reduced hemoglobin and carboxyhemoglobin. Oxalated blood is diluted 1:100 with 0.4 per cent ammonia, reduced with sodium hydrosulfite, and the ratio D_{555}/D_{480} is determined. The per cent saturation of carbon monoxide is read from a prepared graph.

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MEASUREMENT OF SPECIFIC GRAVITIES OF WHOLE BLOOD AND PLASMA BY STANDARD COPPER SULFATE SOLUTIONS*

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The specific gravity measurements are carried out by letting drops of plasma or whole blood fall into a graded series of copper sulfate solutions of known specific gravities, and noting whether the drops rise or fall in the solutions. Each drop, on entering a solution, becomes encased in a sac of copper proteinate and remains discrete, without change of gravity, for 10 or 15 seconds, during which its rise or fall reveals the relation of its gravity to that of the solution. The drops do not have to be of definite size; hence no special pipette is needed for delivering them. No temperature correction is needed, because the temperature coefficients of expansion of the copper sulfate solutions approximate closely those of blood and plasma; regardless of the temperature at which the analysis is made, gravities are given in terms of D_{25}^{25}. The copper sulfate solution automatically cleans itself after each test, because within a minute or two the material of the drop settles to the bottom as a precipitate.

The principle of adding drops of blood to a series of liquids of graded densities and noting whether the drops rise or fall has long been applied, but has hitherto suffered certain limitations. Roy (3) in 1884 used standard aqueous salt solutions to measure the gravity of whole blood, and similar standards were employed for blood by Rogers (4) in his classic studies of dehydration in cholera. The gravity of whole blood can be esti-

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Preliminary publications of the method appeared in the medical journals of the Armed Services (1, 2).

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mated fairly well with standard aqueous solutions of salt or glycerol, but drops of plasma disperse so rapidly in such solutions that accurate observation is difficult. To overcome this difficulty, standards composed of water-immiscible liquids, such as benzene and chloroform, have been used, but such liquids have temperature coefficients that are several times that of plasma or blood, and hence cannot be employed for precise results without accurate temperature control. Furthermore, the organic mixtures that have been generally used are subject to change from different evaporation rates of the components.

The above disadvantages of graded density standards have, in the present method, been overcome by the use of *aqueous standard solutions that coagulate proteins, and that have almost exactly the same temperature coefficients of expansion as blood or plasma of corresponding densities.* The copper sulfate can be replaced by other protein-coagulating solutes, such as zinc sulfate, or sodium chloride plus picric acid.

For measurement of blood and plasma gravities with drop samples, two accurate and elegant methods have been available, the falling drop method of Barbour and Hamilton (5) and the gradient tube method of Jacobsen and Linderstrøm-Lang (6) as applied by Lowry and Hastings (7) and Lowry and Hunter (8). Each of these methods has the advantage over the copper sulfate method of requiring only a single drop of blood or plasma, instead of 3 or 4 drops. The copper sulfate procedure has, however, certain advantages: a stable base is not required (accurate results can be obtained on shipboard), calibration is unnecessary, the apparatus (set of standards) can be supplied ready for operation from central laboratories and can be used without modification of technique, restandardization, or use of temperature corrections, at temperatures from 4–40°. Drops of constant, accurately measured size, such as are required by the Barbour-Hamilton method, are not needed for the copper sulfate procedure, nor is daily restandardization such as is required by the gradient tube.

A stock copper sulfate solution is first prepared which has at 25° a density 1.1000 times that of water at that temperature. From this stock solution the series of standard solutions is prepared by dilution.

PREPARATION OF STOCK SOLUTION OF $D_{25}^{25} = 1.1000$

The stock solution contains, at 25°, 159.0 gm. of $CuSO_4 \cdot 5H_2O$ per liter. At any temperature it contains 5.897 gm. of solvent water per 1 gm. of $CuSO_4 \cdot 5H_2O$.

For preparation of solutions by weight, the sulfate must have the exact composition $CuSO_4 \cdot 5H_2O$, with theoretical H_2O content. The H_2O is determined by heating 2 or 3 gm. of the sulfate in open Pyrex weighing bottles at 300–350° until the weight is constant, which requires 2 or 3 hours. The loss in weight should be 36.06 ± 0.2 per cent. Crystals of U. S. P. sulfate do not usually meet this requirement. "Analytical reagent" grade usually has the correct composition.

The $CuSO_4 \cdot 5H_2O$ and water are mixed in the ratio of 170 gm. of crystals to 1002.5 gm. of water. It is convenient to use sealed bottles containing 170 ± 0.1 gm. of "fine crystals" of analyzed purity.[1]

TABLE I

*Volumes of Water to Add to 170.0 Gm. of $CuSO_4 \cdot 5H_2O$ to Prepare Stock Solution of $D^{25}_{25} = 1.1000$**

Temperature of water	Ml. of water to 170 gm. $CuSO_4 \cdot 5H_2O$
°C.	
10	1003.6
12	1003.8
14	1004.0
16	1004.3
18	1004.7
20	1005.1
22	1005.5
24	1006.0
26	1006.5
28	1007.0
30	1007.7
32	1008.3
34	1008.9
36	1009.6
38	1010.4
40	1011.2

* The solution contains 1002.5 gm. of water per 170 gm. of $CuSO_4 \cdot 5H_2$. The volumes of water given in the table are 0.8 ml. more than theoretical in order to allow for adherence of this amount to the inside of the flask after 2 minutes drainage.

To make 3.2 liters of stock solution, sufficient for a set of 100 ml. standards, transfer three of the 170 gm. portions of sulfate quantitatively to a 4 liter bottle. Fill a 1 liter volumetric flask to the mark with distilled water. Take the temperature of the water. Then add from a burette enough additional water to the liter in the flask to bring the volume up to that indicated in Table I for addition to 170 gm. of sulfate. Empty the water from the flask into the bottle containing the sulfate. Let the water from the upturned flask drain into the bottle 2 minutes. In this manner measure into the bottle one portion of water from the 1 liter flask for each 170 gm. portion of copper sulfate.

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[1] Bottles containing 170.0 gm. of weighed "fine crystals" can be obtained from E. H. Sargent and Company, 155–165 East Superior Street, Chicago 11, Illinois. The $CuSO_4 \cdot 5H_2O$ sold as "fine crystals," smaller than wheat grains, is preferable to that in large crystals, because of the greater ease with which the smaller crystals dissolve.

Particularly if there is any doubt concerning the water content of the $CuSO_4 \cdot 5H_2O$ crystals, it is desirable to check the specific gravity of the solution by a pycnometer with the technique described in the experimental part. If the D^{25}_{25} obtained differs by more than 0.0001 from 1.1000, it is adjusted by adding, per liter of solution, 1 ml. of water for each 0.0001 by which the D^{25}_{25} is too high, or approximately 1 ml. of saturated copper sulfate solution for each 0.0001 by which the D^{25}_{25} is too low.[2]

PREPARATION OF STANDARD COPPER SULFATE SOLUTIONS BY DILUTION OF STOCK SOLUTION

The procedure will be described for making 100 ml. portions of the standard solutions with gravities 0.001 unit apart. To receive the solutions 100 ml. cylindrical prescription bottles with screw caps are cheap and satisfactory, 64 being required for the set. Each bottle is labeled on the side and also marked on the cap with the gravity of the solution that it is to contain, indicated in Table II as D^{25}_{25}. To measure the stock solution it is convenient to have a 25 ml. burette and a 25 ml. pipette attached to the bottle of stock solution by filling devices. A single 100 ml. volumetric flask completes the required apparatus; its mark should not be more than 25 mm. above the level where the neck widens into the bulb.

To prepare each standard the volume of stock solution indicated in Table II is measured into the 100 ml. flask, the volume up to 25 or 50 ml. being measured by pipette, the rest by burette. After the solution is in the flask, water is added in a rapid stream until the surface approaches the neck of the flask. During this addition the water is mixed with the solution by rotating the flask. Then the addition of water is continued, without rotation, until the 100 ml. mark is reached, and the flask is inverted to complete mixing. The preliminary mixing attained before the neck of the

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[2] The density symbol D^{25}_{25} or D^{t}_{t} used in this paper indicates the ratio (weight of solution)/(weight of water), both weights being those of the same volume of fluid at the same temperature when weighed under the same conditions, without correction of either weight for the buoyant effect of air, which would be approximately 1.06 mg. per ml. of fluid. The uncorrected D^{t}_{t} unit is used because it is common in biological work. As is seen from Table IV, the effect of correction for the buoyant effect of air on D^{t}_{t} values within the range of blood and plasma is significant only in the fifth decimal place.

TABLE II

Volumes of Stock $CuSO_4$ Solution of $D^{25}_{25} = 1.1000$ to Dilute to 100 Ml. to Make Standard Solutions for Plasma and Blood

Standard solutions for plasma*		Standard solutions for whole blood*			
Gravity of plasma balanced by standard	Volume of stock solution per 100 ml. standard	Gravity of blood balanced by standard	Volume of stock solution per 100 ml. standard	Gravity of blood balanced by standard	Volume of stock solution per 100 ml. standard
D^{25}_{25}	*ml.*	D^{25}_{25}	*ml.*	D^{25}_{25}	*ml.*
1.015	13.9	1.035	34.3	1.055	54.3
16	14.9	36	35.3	56	55.3
17	15.85	37	36.3	57	56.3
18	16.8	38	37.25	58	57.3
19	17.8	39	38.2	59	58.3
20	18.8	40	39.2	60	59.3
21	19.8	41	40.2	61	60.3
22	20.75	42	41.2	62	61.3
23	21.7	43	42.2	63	62.3
24	22.7	44	43.2	64	63.35
25	23.7	45	44.2	65	64.4
26	24.7	46	45.2	66	65.4
27	25.7	47	46.2	67	66.4
28	26.65	48	47.2	68	67.4
29	27.6	49	48.2	69	68.4
30	28.6	50	49.2	70	69.4
31	29.6	51	50.2	71	70.4
32	30.6	52	51.25	72	71.45
33	31.6	53	52.25	73	72.5
34	32.6	54	53.3	74	73.5
35	33.6	55	54.3	75	74.5
36	34.6				

* For reasons discussed under "Preparation of standard copper sulfate solutions by dilution of stock solution," the standard solutions for plasma are made lighter by 0.0006 gravity unit than the drops of plasma that they balance, and the standard solutions for whole blood are made 0.0001 unit heavier than the drops of whole blood that they balance.

flask is reached is sufficient to prevent measurable shrinkage in the final mixing. The solution is decanted into a labeled bottle.

After the transfer of each standard, the 100 ml. flask is rinsed with water, and is used to prepare the next standard of the series.

For holding the bottles of standards during use a convenient rack can be made from a box about 35 by 60 by 6 cm., with thin crosspieces dividing it into forty-five sections to hold the standards that cover the range ordinarily used, *viz.*, gravities 1.018 to 1.062. At the middle of each end is attached an upright board 6 by 40 cm. The two boards are connected at the top by another which serves as a handle to carry the set, and also serves as a platform on which to place bottles near the level of the eye when they are used for gravity determinations.

When analyses are not numerous, heavy walled test-tubes of not less than 15 ml. inner diameter, filled to within 3 cm. of the top, and closed with rubber stoppers, may be more convenient than bottles as containers of the standard solutions during the analyses.

Provided both stock solution and water are at the *same* temperature when they are mixed according to Table II, the weight ratio of solvent water to copper sulfate is not significantly affected by temperature variations between 10° and 40°. Because the coefficient of expansion of the stock solution is greater than that of water, there is a slight effect, but it is such that a mixture of stock solution and water by volume that would have, when prepared at 25°, a D_{25}^{25} of 1.050, would have, if prepared with the same volumes measured at 10°, a D_{25}^{25} of 1.05005.

The standard solutions prepared according to Table II have slightly different gravities from the D_{25}^{25} values of plasma and blood indicated in Table II as "balanced by" the standards. As shown in the experimental part (Table VI), *plasma* drops balance (neither rise nor fall) in copper sulfate solutions that average lighter than the plasma by 0.0007 gravity unit. Hence, to indicate the correct plasma gravities, the standard solutions for plasma are prepared by Table II to have D_{25}^{25} values 0.0007 ± 0.0001 lighter than the values shown in the first column of the table. Each standard is prepared to have, before it is used, a gravity lower by 0.0006 unit than it is labeled, and during use the gravity falls 0.0002 unit more before the standard is discarded (see "Renewal of standard solutions") so that the D_{25}^{25} range of each standard during the period of its use is 0.0007 ± 0.0001 lower than it is labeled. A plasma of gravity 1.0270 balances in a copper sulfate standard solution marked 1.0270, but the actual gravity of the standard is 1.0263.

Drops of whole blood balance in copper sulfate solutions that on the average have the same gravity as the blood (Table VII). To allow for a decrease of 0.0002 in the gravity of standards for whole blood during use, the standards are made heavier by 0.0001 unit than the D_{25}^{25} values indicated in Table II.[3]

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[3] The dilution table given for preparation of copper sulfate solutions in the preliminary reports of the copper sulfate method (1, 2) gave for both plasma and blood

DRAWING AND PREPARATION OF BLOOD

For samples permitting measurement of the gravity of plasma or serum as well as that of whole blood, procedures are described for venous blood in portions of 1 to 5 ml., and for capillary blood in portions of 0.5 ml. For the gravity of whole blood alone, a procedure for use with finger blood portions of about 0.1 ml. is detailed.

Venous Blood, Portions of 1 to 5 Ml.

Preparation of Oxalated or Heparinized Test-Tubes or Centrifuge Tubes—Test-tubes of heavy walled Pyrex glass or of plastic, of capacity equal to about twice the volume of blood used, are prepared in advance by pipetting into each tube 0.05 ml. of either a 0.4 per cent solution of heparin or a 2 per cent solution of Heller and Paul's (11) oxalate mixture (12 gm. of ammonium oxalate and 8 gm. of potassium oxalate to 1 liter), *for each ml. of blood that the tube is to receive.* The solution is spread in a film over the lower half of the tube, and is dried in an incubator, or in air warmed to not over 60°, or in a vacuum desiccator. Each tube, thus charged with anticoagulant, is provided on the outside with a mark to indicate the volume of blood that it is to receive. It is essential to maintain the above proportion of anticoagulant to blood volume; *viz.*, 1 mg. of oxalate or 0.2 mg. of heparin per ml. of blood. If less anticoagulant is used, coagulation may occur. If more anticoagulant in the form of oxalate is used, it will increase significantly the gravity of the blood and plasma (see Table VIII). *Unmeasured proportions of oxalate must not be used, or gross errors may result in the gravities of blood and plasma, and in hemoglobin and plasma protein concentrations calculated from the gravities.*

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standards 0.0001 to 0.0002 gravity unit lighter than is indicated in these reports, instead of 0.0007 lighter for plasma and identical for blood as in the present paper. Comparison of plasma and blood gravities determined by pycnometer with the gravities of the copper sulfate solutions in which the drops balance (Tables VI and VII) has led to changing the preparation of the standards (Table II) so that they will indicate without correction the blood and plasma gravities determined by pycnometer. The use of the former standards introduced no inaccuracy into the calculation of plasma protein and blood hemoglobin concentrations, because the formulas for the calculations were based on comparisons of protein and Hb concentrations with gravities estimated by the same copper sulfate standards. Laboratories using the former standards, and the formulas and nomograms developed for calculations of plasma proteins (1, *b*) and blood Hb from gravities obtained with them, can continue to do so without error. If the present standards are used, however, the formulas and nomogram developed in the succeeding papers (9, 10) are to be employed. Use of the former formulas with gravities determined by the present standards would cause errors of about +0.2 gm. of calculated plasma protein per 100 ml. of plasma and −0.2 gm. of Hb per 100 ml. of blood.

With regard to choice between heparin and oxalate, heparin is preferable if the gravity measurements are to be made within a few hours, because heparin does not measurably affect the gravity. If heparinized blood stands till the next day, however, small clots may form that interfere with the gravity measurement. Also, *in some specimens of heparinized blood that have stood more than 18 hours in the refrigerator, some change has been seen to occur as a result of which drops of the blood, when let fall into copper sulfate solution, fail to break through the surface film of the solution*, but remain attached to the film by streamers which prevent observation of the gravity. This phenomenon has not been observed in oxalated blood, nor in freshly heparinized blood.

Drawing Venous Blood—Tourniquets should not be applied longer than 1 minute. Longer applications may force so much fluid out of the blood that concentrations of both plasma protein and blood hemoglobin are measurably increased.

Handling Venous Blood; without Anticoagulant—If the specific gravity of whole blood only, or of whole blood and serum, is to be measured, the blood is drawn into a syringe, and drops are at once delivered from the needle into the copper sulfate solutions to determine whole blood gravity. Then the remainder of the blood may be transferred to a centrifuge tube and allowed to clot in order to obtain serum.

With Anticoagulant—If results for whole blood and plasma are desired, a measured amount of anticoagulant is added, as described above, to the tube that is to receive the blood. The gravity of the whole blood may be determined either immediately after drawing, by dropping blood from the syringe needle into copper sulfate standards, or after transfer to the oxalated or heparinized tube, before centrifugation. In the latter case *it is necessary to mix the cells and plasma thoroughly* before the blood sample is withdrawn from the tube into a dropper or syringe. To attain complete mixing either invert the tube containing the oxalated or heparinized blood ten times, or stir with a glass rod with a mushroom end, which is raised and lowered through the blood ten times, just before the sample is drawn into the dropper or syringe. Gross error in the estimation of blood gravity and in gravity-calculated hemoglobin could result if the blood sample were taken from blood in which partial settling of the cells had occurred.

Capillary Blood, 0.5 Portions for Gravity of Both Whole Blood and Plasma[4]

A small test-tube is prepared, as shown in Fig. 1, with a rubber cap through which pass two short capillary tubes of about 1 mm. bore. The longer capillary serves to admit blood, the other to let out the replaced air. The inlet capillary should be cleaned with chromic acid before it is placed in the rubber cap; otherwise blood may not readily flow through it. The test-tube is marked at the level indicating 0.5 ml., and is coated within by a dried film from 0.025 ml. of the heparin or oxalate solution described above for venous blood.

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[4] Procedure devised by L. E. Farr (personal communication). The rubber caps are made from stoppers catalogued as No. 2319-B by the Arthur H. Thomas Com-

A deep cut is made in a finger, or on the back of a thumb just below the nail, and the exuding blood is drawn by capillary attraction into the long inlet capillary, while the test-tube is held at sufficient downward slant to cause the blood to drop down into the tube. As soon as 0.5 ml. of blood has been collected, it is well stirred with a small rod, and a portion is withdrawn by a small syringe or medicine dropper, and used to determine the whole blood gravity.

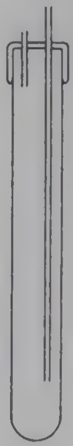

FIG. 1. Farr tube, 6 by 50 mm. outside measurement, arranged to receive 0.5 ml. of capillary blood, for specific gravity of both blood and plasma.

The remainder of the blood is centrifuged, and the supernatant plasma similarly withdrawn for gravity estimation.

Capillary Blood, 4 or 5 Drops, for Gravity of Whole Blood

Glass capillary tubes, of 1 to 1.5 mm. bore and about 150 mm. in length, are used.[5] It is convenient, for expulsion of blood drops, to provide the

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pany for serum bottles. The portion of stopper that is for insertion into a bottle is cut off to form the cap shown in Fig. 1. The cap is pierced with a needle to provide holes to receive the capillary tubes. The test-tubes are fermentation tubes, and are catalogued by Thomas as No. 9446, thick wall, without lip, 50 by 6 mm. outside dimensions.

[5] Obtainable from the Kimble Glass Company, Vineland, New Jersey. Capillaries sold for melting point tubes may also be used.

tubes with small rubber bulbs, shown in Fig. 2, open at both ends, used with "vaccine tubes."[6] *The bores of the glass capillaries must be clean* in order to draw in blood by capillary attraction. The tubes can be cleaned by soaking them in chromic-sulfuric acid cleaning mixture in a test-tube, then rinsing twenty times with distilled water, and drying in an oven.

The rubber bulbs shown in Fig. 2 are convenient, but not necessary. Without them one can expel the greater part of the blood from a filled capillary by raising it upright, and the last drop can be expelled by gentle pressure of the breath.

A finger or ear lobe is cleaned with 70 per cent alcohol, wiped perfectly dry with sterile gauze or cotton, and pricked with a lancet. The cut must be deep enough to yield a large drop of blood. The size of the drop may be increased by massaging the finger from the base towards the tip. One end of a glass capillary is immersed in the drop while the capillary is held horizontally. If the bore is clean, the first half will fill without tilting the tube downwards at all. To fill the last half it is necessary to slant the tube slightly downwards from the finger (Fig. 2), but as little slant as possible is used or a bubble may be drawn in.

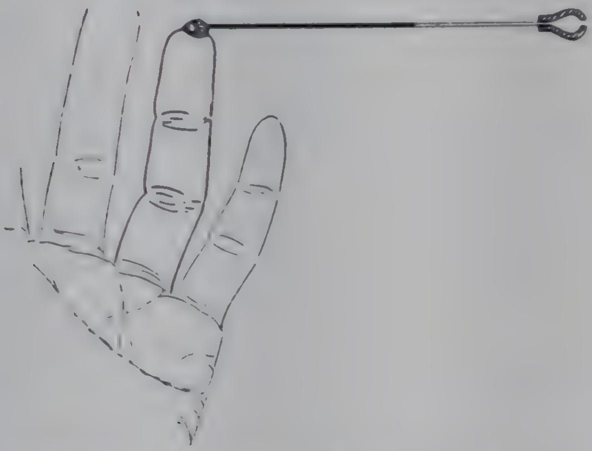

FIG. 2. Drawing capillary blood from finger for specific gravity of whole blood

By vaselining slightly the delivery end before the drops are delivered, the size of the drops may be diminished so that 1 or 2 more drops from the tubeful are obtained. The blood is immediately used for the gravity test, before there is time for clotting to begin. It serves for approximate hemoglobin estimations (10).

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[6] The bulbs are obtainable from the West Rubber Company, 117 Shackamaxon Street, Philadelphia, Pennsylvania.

SPECIFIC GRAVITY DETERMINATION

Procedure

The drop of serum, plasma, or whole blood is delivered from a height of about 1 cm. above the solution[7] from a medicine dropper, a capillary, or a syringe needle. It is preferable to use small drops for the reason that they permit more tests before the standard solution must be changed. There-

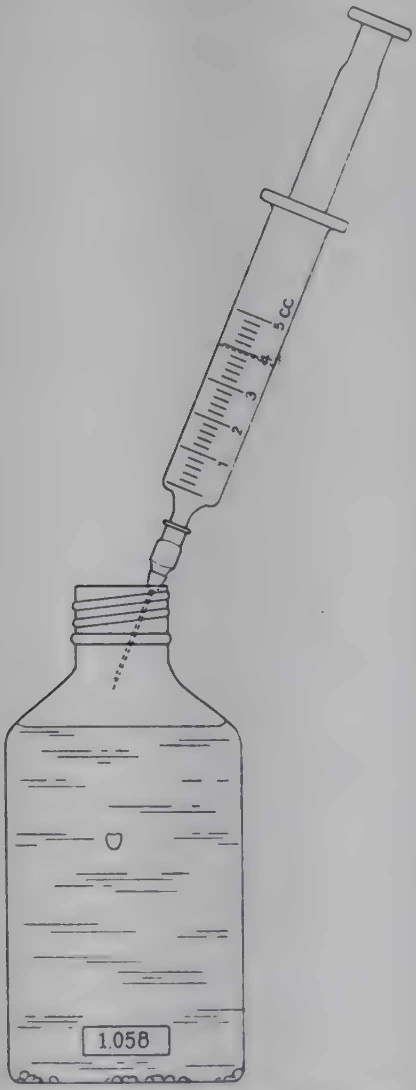

Fig. 3. Dropping blood into a standard copper sulfate solution

fore a syringe needle serves well, and a medicine dropper with a fine tip is preferable to one with a coarse tip.[8] Greasing the outside of the tip with vaseline also reduces the size of the drop. When the drop is delivered, it is convenient to steady the syringe or dropper on the edge of the bottle (Fig. 3).

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[7] If the drop strikes with too little force, it may not break through the surface film. On the other hand, if the drop falls from too great a height, it may be broken up on striking the solution, or its momentum may carry it too far below the surface; *the momentum should be only enough to carry a drop, of gravity slightly less than that of*

The delivered drop breaks through the surface film of the solution and its momentum carries it 1 or 2 cm. below the surface; within 5 seconds the momentum of the fall is lost, and the drop then either begins to rise, becomes stationary, or continues to fall. The gravity of the drop relative to that of the solution does not change appreciably until the drop has been immersed in the solution for another 10 or 15 seconds, and there is ample time to note its behavior during this interval. If the drop is lighter than the test solution, it will rise, perhaps only a few mm., and may begin to sink immediately afterward. If the drop is of a gravity to balance in the standard test solution, it will become stationary for a few seconds and then fall. If the drop is heavier, it will continue to fall during the entire interval. *In summary, the behavior during the 10 seconds after the drop has lost the momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution; if it rises at all during this period, it is lighter than the standard.*

The following order of using the solutions with plasma is usually followed. Place the first drop in the 1.027 standard, with the gravity of average normal plasma. According to whether the drop sinks or rises, place the second drop four points away, at 1.031 or 1.023. If the plasma gravity is within the range 1.023 to 1.031, test intermediate points between the first two as in the example given below. If the plasma gravity is outside the range 1.023 to 1.031, make the second test at 1.019 or 1.035.

A similar order is followed with whole blood, the first test being made with the standard of gravity 1.056 if the blood is a woman's, 1.059 if a man's. If it is known in advance that the subject is low in plasma proteins or hemoglobin, the initial test may be made with a standard of lower gravity; *e.g.*, 1.023 for plasma, 1.050 for blood.

Example—The following example shows how, by bracketing on the probable extremes of a plasma's gravity range and then testing intermediate points, one can with 4 drops estimate the specific gravity with a precision that can be duplicated within 0.0002 unit.

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the solution, 1 or 2 cm. below the surface. A fall of about 1 cm. usually gives the right striking force. If room temperature is above 30°, a fall of less than 1 cm. suffices, since both the viscosity of the solution and the strength of the surface film are less than at lower temperatures.

[8] When a medicine dropper is used, it is convenient to apply a screw clamp to the rubber bulb. By manipulating the screw one can easily draw in a sample of desired volume, and expel a series of drops without forming bubbles by admitting air into the tip after expulsion of any drop.

4 successive drops gave the following results, in which the figures indicate the gravities of the standards, and plus or minus after each gravity figure indicates that the plasma gravity was greater or less than that indicated by the preceding figure: 1.027 +, 1.031 −, 1.029 +, 1.030 −. The plasma gravity was greater than 1.029 and less than 1.030, and could therefore be placed at 1.0295, within less than ±0.0005.

By noting the relative rate of fall or rise in the two adjacent solutions, 1.029 and 1.030, it was further obvious that the plasma was nearer 1.029 than 1.030. Being less than 1.0295 and greater than 1.0290, it could be placed at 1.0293, within ±0.0002.

Special Points and Precautions

1. Surface Film Effects in Analyses—Occasionally a drop will fail to make a clean break through the surface of the copper sulfate solution, and remain attached by a tentacle to the film. In this case the drop is lifted from the surface by a rod or stick, or is made to sink by tapping the tube, and a fresh drop is tried.

After each test, one makes sure that the surface film is left clean and free from fragments. If any are left on the film, they are likely to prevent a clean break through of the drop in the next test. Fragments caught in the surface film can usually be detached by tapping the tube; they then sink to the bottom. Sometimes, however, a fragment of fatty nature or holding a bubble will continue to float on the surface. Such fragments are removed with a rod or stick.

2. Avoidance of Bubbles—Even a minute air bubble attached to a drop of blood or plasma will make it float. While drawing finger blood into a capillary tube (Fig. 2), make sure that the tip is kept immersed, so that no air bubbles will be drawn in, and hold the tube horizontal or at only a slight downward slant from the finger. When a drop is expelled into copper sulfate solution, enough excess blood or plasma should be present to leave some in the dropper. After releasing a drop from a medicine dropper without a screw clamp, one is likely to draw into the tip a bubble, which may attach itself to the next drop; the use of a syringe, or of a medicine dropper provided with a screw clamp, eliminates this source of error.

3. Temperature—The temperature may vary from 4–40° without significantly affecting results, so long as the copper sulfate solutions and the blood or plasma are within 5° of the same temperature.

The change of gravity per 1° change in temperature is about 0.0003. However, the error caused by initial difference in temperature between the blood or plasma and the standard copper sulfate solution is much less, because when a drop of blood or plasma enters the copper sulfate solution the drop approaches the temperature of the solution so rapidly that the effects of several degrees of initial difference are nearly eliminated. Thus when plasma at 16° was dropped from a syringe (small drop) into copper sulfate solution at 26°, the error in estimating the gravity of the plasma was only 0.0004, or one-eighth of what it would have been if the drop had not moved its temperature towards that of the solution. When 5 ml. of blood at 37° were drawn from a vein into a 10 ml. syringe at 26°, and at once tested with copper sulfate solutions at 26°, the gravity of the blood was never found to be more than 0.0002 lighter than when the test was made after the blood had cooled to 26°.

Blood drawn from a vein into a syringe can be delivered at once from the needle of the syringe into standard solutions at 20°, or above, without error from temperature ordinarily exceeding 0.0003 in the gravity measurement. However, blood or plasma drawn into a medicine dropper and delivered into the copper sulfate solutions should be within 5° of the temperature of the solutions. Blood from a refrigerator should be warmed to room temperature before the gravity is measured.

If cold copper sulfate standard solutions are brought into a warmer room, let the solutions come to within 1° or 2° of room temperature and then shake vigorously for a few seconds before using them. This precaution is necessary for three reasons: (1) The difference in temperature between the solutions and the blood or plasma may exceed the permissible 5°. (2) If the solutions are used while they are warming, they may develop convection currents that interfere with the determinations. (3) The solutions when cold dissolve an amount of air that makes them supersaturated when they are warmed. The warming can be accelerated by immersing the standards in water a few degrees above room temperature. The solutions are shaken *after* warming to discharge the excess of dissolved air. Otherwise air bubbles might form as the solutions stand, and attach themselves to drops of blood or plasma dropped into the solutions.

As precautions against convection currents, do not leave the standard solutions near a stove, on a window-sill, etc. If a container is held in the hand during a determination, hold it only by the top in order to avoid warming the solution with the hand.

4. Renewal of Standard Solutions—A 100 ml. standard solution should be replaced by a fresh solution after receiving 50 drops of blood or plasma, if the error in measurement of blood or plasma gravity due to the standard is to be kept within ±0.0001 gravity unit. Tests have shown that addition of 50 small drops, about 1.5 ml., of blood or plasma to 100 ml. of standard $CuSO_4$ solution, causes the gravity of the copper sulfate standard solution to decrease by about 0.0002 unit. The standard solutions prepared according to Table II are made 0.0001 gravity unit too high to give a correct average plasma or blood gravity when used fresh. After receiving 50 drops, the standards become 0.0001 gravity unit too low. The resultant error in blood or plasma gravity is in the direction opposite to the deviation of the standard, being −0.0001 with a fresh standard and +0.0001 with a standard that has received 50 drops. The ±0.0001 error in gravity corresponds to ±0.04 gm. of protein per 100 ml. of plasma or ±0.06 gm. of hemoglobin per 100 ml. of blood, which is ordinarily negligible.

It is desirable to paste on each standard tube or bottle a piece of cross-section paper containing a number of squares equal to the number of drops of blood or plasma that the solution can receive. Mark off with a pencil one square for each drop added, and replace the solution when all the squares have been marked off.

Rough controls can be made by adding 1.5 ml. of plasma to a 100 ml. standard of 1.027 gravity and 1.5 ml. of blood to a 100 ml. standard of 1.058 gravity. The amount of copper proteinate precipitate that settles to the bottom of the controls indicates the maximum permissible.

If a maximal error corresponding to +0.12 gm. of plasma protein, or +0.18 gm. of hemoglobin, per 100 ml., from overuse of the standard solutions is permissible, the number of drops of blood or plasma permissible is raised to 100 per 100 ml. of standard solution; the effect is to decrease the gravity of the standard by about 0.0004 unit before it is discarded, with a resultant error of +0.0003 in the last blood or plasma gravity measurements made.

If, for any reason, it is impossible to renew a standard solution after it has been used often enough to decrease its gravity by 0.0002 or 0.0004, the standard may be brought back to approximately its original gravity by adding 0.2 or 0.4 ml., respectively, of stock $CuSO_4$ solution per 100 ml. of standard. Or, if stock solution is not available, the standard may be relabeled; *e.g.*, a 1.0270 standard is relabeled 1.0268 or 1.0266. The authors do not recommend either subterfuge, but they may be resorted to in an emergency.

Although a standard of 100 ml. volume should be replaced after receiving 50 or 100 drops of blood or plasma, only a few standards of a set need be replaced after 50 or 100 blood analyses, because only those within the normal range are used in most analyses. Standards outside the normal range, 1.025 to 1.029 for plasma and 1.052 to 1.064 for whole blood, will ordinarily require infrequent replacement.

5. Avoidance of Evaporation of Standard Solutions—Unless the standard solutions are tightly closed when not in use, evaporation may occur to such an extent that the blood and plasma gravity results obtained are erroneously low. The bottles or tubes of the standard solutions are opened only for the length of time required for determination and then are closed tightly. For each determination, only those standards are opened that are likely to be used.

6. Correction for Added Anticoagulants—No corrections are needed if heparin, 0.1 or 0.2 mg. per ml. of blood, is used.

For each mg. of the ammonium-potassium oxalate mixture added per ml.

of blood, subtract 0.0004 from the observed G_B and the observed G_P (see Table VIII). If a test-tube prepared with 5 mg. of ammonium-potassium oxalate receives the expected 5 ml. of blood, the correction to G_P and G_B is therefore 0.0004. Neglect of the 0.0004 correction would lead to an overestimation of the plasma proteins by 0.15 gm. per 100 ml. of plasma, and of hemoglobin by 0.1 gm. per 100 ml. of blood, errors which may for some purposes be neglected.

If, however, the volume of blood placed in the oxalated test-tube prepared to receive 5 ml. is less than 5 ml., the oxalate concentrations will be greater, and the corrections to G_P and G_B will be as follows: for 4 ml. of added blood, −0.0005; for 3 ml., −0.0007; for 2 ml., −0.0010; for 1 ml., −0.0020.

7. Standard Solutions Carried to High Altitudes—Dr. R. A. Johnson (personal communication) reports that when the standard copper sulfate solutions are carried from a low to a high altitude they must be shaken before being used, to discharge the dissolved air with which they are supersaturated. Otherwise air bubbles may form, as when cold solutions are warmed, and interfere with the gravity measurements.

8. Material to Which Copper Sulfate Method Is Inapplicable—As mentioned before, heparinized blood, or its plasma, after standing over 18 hours in the ice box may become so altered in surface properties that drops will not break through the surface film of the copper sulfate solution.

Quigley and Muraschi (12) found that two sera which had been preserved for 5 years failed to form copper proteinate sacs when dropped into copper sulfate solutions. They attributed this behavior to the formation of amino acids in the sera, which contained 46 and 105 mg. of α-amino nitrogen, respectively, per 100 ml. by the ninhydrin-CO_2 method (13), about 10 and 25 times the normal amount. Addition of similar amounts of amino acids to normal serum had a similar effect.

The method is not applicable to solutions containing less than about 2 gm. of protein per 100 ml., hence cannot be used for transudates and exudates.

9. Material Other Than Blood and Plasma to Which Copper Sulfate Method Is Applicable—Presumably the procedure can be used with any aqueous solution containing enough protein to form an adequate copper proteinate sac. Dr. Eugene Opie (personal communication) has employed it with pieces of tissue to estimate changes in water content.

EXPERIMENTAL

Relation of Specific Gravity to Concentration in Copper Sulfate Solutions

Solutions were prepared by weight. Into 50 ml. flasks were weighed varying amounts of $CuSO_4 \cdot 5H_2O$, the composition of which had been checked by drying 3 gm. samples at 300° to constant weight. 40 ml. of water were then pipetted into each flask, and the flasks were weighed again. The flasks were stoppered and rotated by hand till the copper sulfate was dissolved.

For the density determinations a pycnometer of 12.8 ml. capacity of the Ostwald-Sprengel type, of Pyrex glass, was used. Its form was that shown in Fig. 4 with the following changes: The horizontal arms were each 40 mm. long; no cock was attached to *A*; an etched ring encircled the arm *B* at about 25 mm. from its end. The pycnometer filled with water or solution was placed in a stirred water bath at 25° ± 0.05° until constancy of the location of the meniscus in the marked arm showed that constant temperature had been reached, which required about 5 minutes. The meniscus was then brought to the mark by touching the outlet of the unmarked arm with a pointed strip of blotting paper. The meniscus was observed with a lens, and if it was not exactly on the mark, a correction of 0.7 c.mm. per mm. of displacement was made. The pycnometer was then wiped dry and weighed. During weighing the pycnometer was suspended from the balance hook at such an angle that the meniscus retreated about 15 mm. from the end of capillary *A;* with this precaution no loss of weight could be detected during 30 minutes in the balance case. To prevent error from static charge on the wiped pycnometer (which on a dry day could be several mg.) two copper plates painted with radium bromide to ionize the air were placed in the balance case. With these, static error disappeared within about 1 minute. Before weighing a series of copper sulfate solutions, the empty pycnometer was immersed in the bath, wiped, and weighed in the same manner. Before and after a series of weighings of copper sulfate solutions, water at 25° was weighed in the pycnometer. Weights checked by the Bureau of Standards were employed. Duplicate weighings usually agreed within 0.2 mg. Correction for the buoyant effect of air was made by adding, to the uncorrected weight of the fluid in the pycnometer, 1.06 mg. per ml. of fluid. Variations in the correction due to atmospheric conditions were made negligible by the daily check with water.

The results were plotted on large scale coordinate paper with values of the ratio, $R = (D_{25}^{25} - 1):C$, as ordinates and D_{25}^{25} as abscissae, C being the gm. of $CuSO_4 \cdot 5H_2O$ per 100 gm. of solution. The small variation of R with the density made graphic estimation of R for a given density possible to 1 part per 5000, whence C could be calculated as $R/(D_{25}^{25} - 1)$. The results are given in Table III, which served as the key table for computation of Table II.

The values of D_4^{25} of Table III were compared with values interpolated from the data in the International Critical Tables (14), and showed agreement within 1 part in 500 in the concentrations of copper sulfate of the indicated densities.

To check the accuracy of the procedure previously described for preparing standard solutions by dilution of the stock solution, volumes of the latter from 10 to 80 ml. were measured into 100 ml. flasks from pipettes calibrated to 1 per 1000 accuracy, and water was added to the 100 ml. mark in the manner described for preparation of standard solutions. The densities were measured by pycnometer, as described above, and interpolated on the curve obtained from solutions prepared by weight. The interpolation indicated agreement, with solutions prepared by weight, within the limits of error of the measurement; *i.e.*, the technique described for preparing the standard solutions does not involve measurable volume error from incomplete mixing during addition of the water.

TABLE III

Relation of Concentration to Density in Copper Sulfate Solutions at 25°

Density of standard solutions			$CuSO_4 \cdot 5H_2O$ per 100 gm. solution	$CuSO_4$ per 100 ml. of solution $= c \times d$	Stock solution per 100 ml. standard solution $= \frac{e}{0.1590^*}$
D^{25}_{25}		$D^{25}_{4} = \frac{b}{0.99707}$			
Uncorrected for buoyant effect of air	Corrected for effect of air				
(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)
			gm.	*gm.*	*ml.*
1.010	1.00999	1.00703	1.520	1.531	9.63
20	1.01998	1699	3.031	3.082	19.38
30	2997	2699	4.523	4.646	29.22
40	3996	3695	6.001	6.222	39.13
50	4995	4691	7.465	7.814	49.13
60	5994	5683	8.910	9.415	59.21
70	6993	6679	10.53	11.02	69.31
80	7992	7675	11.74	12.64	79.50
1.100	1.09990	1.09667	14.50	15.90	100.0

* The stock solution at 25° contains 0.1590 gm. of $CuSO_4 \cdot 5H_2O$ per ml.

Temperature Coefficients of Expansion of Plasma and Blood, and of Copper Sulfate Solutions of Similar Specific Gravities

That no temperature corrections are required in determinations of blood and plasma gravities by the copper sulfate method is due to the fact that blood and plasma have temperature coefficients nearly identical with those of copper sulfate solutions of similar gravities.

The coefficients of expansion of plasma, whole blood, and copper sulfate solutions were determined in a dilatometer of about 18 ml. capacity and of the form shown in Fig. 4. The left arm is 4 cm. long and has an etched mark at *A*. The right arm, *B*, is 40 cm. long and has a scale indicating volumes of fluid held between points on the scale and mark *A*. Both arms are at the same level to equalize hydrostatic pressure and are joined by a solid glass bridge to give strength and rigidity. The scale on *B* is of sufficient length to read volume changes over the temperature range 10–40°. The scale on *B* was calibrated with mercury.

The apparatus was filled by dipping the end of *A* into the liquid (which was at room temperature, 22–26°) and applying suction to the end of *B* till the fluid meniscus lay approximately in the middle of the scale. *A* was then wiped dry and a short length of rubber tubing bearing a glass stopcock and screw clamp, as shown in Fig. 4, was slipped over the end. When the cock was closed, the meniscus in *A* was adjusted to the etched mark by turning the screw clamp. The bulb of the dilatometer was then immersed in the center of a water bath of about 9 liters capacity to the depth shown by the dotted line, and supported so that the two arms were horizontal. A standard thermometer was placed alongside the dilatometer, with the center of the thermometer bulb at the same level as the center of the dilatometer bulb.

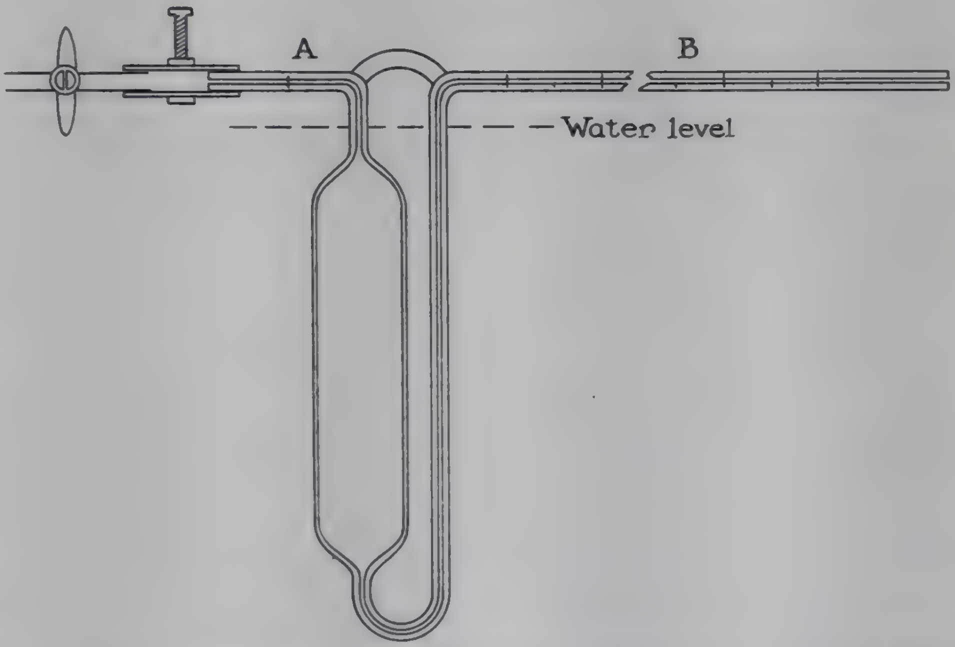

FIG. 4. Dilatometer for measurement of coefficient of expansion of fluids

To determine the coefficients of expansion over the range 40–25°, the bath was warmed to 40° and maintained at that temperature for about an hour, till the fluid in the pycnometer had come to thermal equilibrium.

The heat was turned off and after about 15 minutes the meniscus in the arm *A* was adjusted to the mark, the volume was read off the scale on arm *B*, and the temperature was read on the thermometer. Temperature and volume were then read together every 20 to 30 minutes, the meniscus in *A* being adjusted to the etched mark when necessary, immediately before each reading; readings were continued till the temperature had fallen to 25°. For the range 10–25°, the bath containing the pycnometer was cooled by the addition of ice to 8° or 9°, maintained there for about an hour, and then allowed to warm up to room temperature. Readings of volume and temperature were started when the temperature had risen to 10°. About 6 hours were taken for the temperature of the bath to fall from 40° to 25°, and about the same interval to rise from 10° to 25°. Variations in room temperature were insufficient to cause, in the relatively small volume of liquid in the capillary arms, volume changes that were significant in relation to the changes of the total fluid in the dilatometer.

TABLE IV

Coefficients of Expansion of Water, Plasma, Blood, and of Copper Sulfate Solutions of Specific Gravities Approximating Specific Gravities of Plasma and Blood

Temperature interval	Mean coefficient of expansion over temperature interval; ml. increase in volume of 1 ml. caused by temperature rise of 1°				
	Water	Plasma of D^{25}_{25} 1.0264	Copper sulfate solution of D^{25}_{25} 1.0270	Blood of D^{25}_{25} 1.0569	Copper sulfate solution of D^{25}_{25} 1.0597
°C					
10–15	0.00012	0.00017	0.00016	0.00021	0.00021
15–20	18	23	21	25	25
20–25	24	27	26	29	29
25–30	28	31	30	32	32
30–35	33	35	34	36	36
35–40	37	39	38	40	40

From the fact, shown in Table IV, that the technique gave, within the limits of its precision, temperature coefficients for water which agreed with those in the International Critical Tables, it appears that errors, from lag in temperature change of the dilatometer behind the change in the bath, and from other sources, were negligible in the fifth decimal place.

Non-Effect of Temperature on Results of Plasma and Blood Specific Gravity Determinations by Copper Sulfate Method

Specific gravity determinations were performed by the method described on a sample of blood and one of plasma at room temperature, and were then duplicated in rooms at 4° and at 38°, the samples and standard copper sulfate solutions being left in each room long enough to come to within 1° of its temperature. In order to check the readings to the fourth decimal place, after each gravity had been estimated to the fourth place by the usual interpolation method, with use of copper sulfate standards graded at intervals of 0.001, a mixture of the nearest two standards was made, such that the mixture had exactly the estimated blood or plasma gravity, and the blood or plasma was checked with this standard. Results are given in Table V.

TABLE V

Non-Effect of Temperature on Results of Plasma and Blood Gravities Estimated by Copper Sulfate Method

Temperature	Gravities found by copper sulfate method	
	Blood	Plasma
°C.	D_{25}^{25}	D_{25}^{25}
4	1.0585	1.0255
23	1.0585	1.0252
35	1.0588	1.0252

Comparison of Blood and Plasma Gravities Measured by Pycnometer with Gravities Measured by Copper Sulfate and Gradient Tube Methods

The pycnometer measurements were made as described above for the copper sulfate solutions of Table III, except that the measurements were carried out in a room at approximately 25°, and a water bath was not used. All fillings and weighings were carried out in duplicate or triplicate. The temperature of the balance case was maintained usually at 25° ± 1°. If the temperature of the test solution was not identical with that of the water, the observed weight of the water was corrected for the temperature difference, which was always slight. The specific gravity was calculated directly from the weights as D_{25}^{25} = (weight of test solution) /(weight of water), both weights being the apparent weights, uncorrected for the buoyant effect of air.

Duplicate weighings routinely agreed to ±0.1 mg. Measurements of specific gravity routinely agreed within ±0.00002 unit.

For the gradient tube method two 500 ml. cylinders were prepared as described by Lowry and Hunter (8), one for the range of plasma and one for whole blood. The only difference introduced from the technique of Lowry was that the standard copper sulfate solutions of D_{25}^{25} accurate to

±0.0001 (Table III) were used to standardize the gradient tubes, instead of the potassium sulfate solutions used by Lowry. In order to assure maximal accuracy, when the gravity of a blood or plasma had been measured, the accuracy of the standardization curve of the gradient tube at that point was rechecked by 2 drops of copper sulfate solution within 0.001 of the determined gravity. Readings on the gradient tube were reproducible to ±0.0001, and deviations from true gravities exceeding this limit could be attributed to factors, such as absorption of bromobenzene by the drops of blood or plasma, other than the precision of the readings.

The copper sulfate method was carried out as described in the preceding pages, except that the standard solutions were graded at intervals of 0.0005, instead of the 0.0010 intervals used in routine analyses. The standards were prepared from Table III, not from Table II. The stock solution was prepared by weight from analyzed $CuSO_4.5H_2O$; both it and some of the standards were repeatedly checked by pycnometer and the D_{25}^{25} values were found exact within ±0.0001. Duplicate gravity estimations of the plasma and blood drops could be checked within 0.0001, and deviations from true gravities exceeding this limit could be attributed to factors other than the precision of the readings.

The results obtained with twenty-two samples of plasma are given in Table VI, and results with whole blood from twenty of the same subjects in Table VII. It is evident from comparison with the pycnometer values that readings by both the copper sulfate method and the gradient tube are slightly but measurably affected by sources of error other than the precision of the readings. In the copper sulfate readings, drops of plasma were balanced in solutions that averaged 0.0007 unit lighter than the plasma. When correction was made for this mean difference, the standard deviation of the corrected copper sulfate readings from the pycnometer values was ±0.0003. The gradient tube readings, on the other hand, indicated gravities averaging 0.0002 heavier than the pycnometer values; when correction was made for this mean difference, the standard deviation of the corrected gradient tube readings from the pycnometer values was ±0.0003, the same as for the corrected copper sulfate readings. The standard deviation of ±0.0003 corresponds to ±0.1 gm. of protein per 100 ml. of plasma.

Lowry and Hunter (8), working with serum (ten samples) and Hoch and Marrack (15) with plasma found that serum and plasma drops balanced in copper sulfate solutions that averaged 0.0010 lower than the plasma or serum gravity measured by pycnometer. Lowry and Hunter (8) found that their gradient tube method indicated serum gravities 0.0005 higher than by pycnometer. The deviations from pycnometer serum values indicated by our results are, for both the $CuSO_4$ and the gradient method, in

TABLE VI

Comparison of Plasma Gravities (D^{25}_{25}) Measured by Pycnometer, Gradient Tube, and Copper Sulfate Methods

Sample No.	Values of D^{25}_{25} measured by three methods						
		Copper sulfate method			Gradient tube		
	Pycnometer (a)	D^{25}_{25} of solution balancing plasma* (b)	D^{25}_{25} of plasma estimated as $b + 0.0007$ (c)	Deviation, $d = c - a$ (d)	Uncorrected* (e)	Corrected by subtracting 0.0002 (f)	Deviation, $d = f - a$ (d)
1†	1.0200	1.0195	1.0202	+0.0002			
2†	233	226	233	0	1.0233	1.0231	−0.0002
3	247	247	254	+ 7	255	253	+ 6
4†	248	241	248	0	249	247	− 1
5	256	251	258	+ 4	254	252	− 4
6	257	254	261	+ 4	262	260	+ 3
7	259	253	260	+ 1	262	260	+ 1
8†	262	255	262	0	264	262	0
9	264	254	261	− 3	260	258	− 6
10	264	258	265	− 1	267	265	+ 1
11	267	259	266	− 1	270	268	+ 1
12	267	255	262	− 5	270	268	+ 1
13	270	263	270	0	270	268	− 2
14	271	263	270	− 1	270	268	− 3
15	272	265	272	0	278	276	+ 4
16	273	264	271	− 2	279	277	+ 4
17	276	271	278	+ 2	276	274	− 2
18	276	270	277	+ 1	280	278	+ 2
19	277	265	272	− 5	277	275	− 2
20	278	271	278	0	279	277	− 1
21	287	276	283	− 4	288	286	− 1
22*	401	399	406	+ 5			
Mean deviation from pycnometer		−0.00070	0.0000	0.0000	+0.00021	0.0000	0.0000
Standard deviation, $\sqrt{d^2/(N-1)}$				±0.00029			±0.00029

* Measured as described by Lowry and Hunter (8) except that the calibration curve of the gradient column was made with drops of copper sulfate solution of known D^{25}_{25} instead of with drops of standard K_2SO_4 solution.

† Samples 1, 2, 4, 8, and 22 were from patients. The other seventeen plasmas were normal.

the same directions as Lowry and Hunter's but not quite so great. Hoch and Marrack suggest that enclosure of a film of air about the plasma drop may cause it to appear lighter than the copper sulfate solution, and state that when they delivered the plasma under the solution, instead of letting the drop fall through the air, the difference disappeared.

TABLE VII

Comparison of Specific Gravities (D^{25}_{25}) of Whole Blood Measured by Pycnometer, Gradient Tube, and Copper Sulfate Methods

Sample No.	Values of D^{25}_{25} measured by three methods					
		Copper sulfate method		Gradient tube		
	Pycnometer (*a*)	D^{25}_{25} of solution balancing blood (*b*)	Deviation, $d = b - a$ (*d*)	Uncorrected* (*e*)	Corrected by subtracting 0.0003 (*f*)	Deviation, $d = f - a$ (*d*)
2†	1.0464	1.0471	+0.0007	1.0475	1.0472	+0.0008
3	554	554	0	556	553	−1
4†	493	490	−3	497	494	+1
5	566	564	−2	566	563	−3
6	534	536	+2	538	535	+1
7	587	580	−7	582	579	−4
8†	512	516	+2	518	515	+3
9	572	571	−1	575	572	0
10	563	558	−5	563	560	−3
11	581	584	+3	583	580	−1
12	605	607	+2	608	605	0
13	605	600	−5	606	603	−2
14	599	599	0	601	598	−1
15	589	592	+3	592	589	0
16	599	605	+6	609	606	+7
17	537	540	+3	539	536	−1
18	537	541	+4	543	540	+3
19	579	585	+6	581	578	−1
20	551	550	−1	552	549	−2
21	563	558	−5	563	560	−3
Mean deviation from pycnometer		+0.00004	0.0000	+0.00028	0.0000	0.0000
Standard deviation, $\sqrt{d^2/(N-1)}$			±0.00038			±0.00031

† See the foot-notes of Table VI. The numbers in the left-hand column indicate bloods from which plasmas with the same numbers in Table VI were obtained.

Drops of whole blood (Table VII) balanced in copper sulfate solutions of the same average gravity as the blood. The consistent difference from the gravity of the balancing copper sulfate solutions, noted in plasma, is not apparent in whole blood.

Effects of Oxalates Added to Whole Blood on Specific Gravity of Blood and of Plasma Separated from Blood

Ammonium oxalate, potassium oxalate, and Heller and Paul's mixture (3 parts by weight of ammonium oxalate to 2 parts of potassium oxalate) were added to blood in amounts varying from 1 to 10 mg. per ml. The specific gravities of the bloods, and also of the plasma separated by centrifugation, were then measured by the copper sulfate method. The effects of the oxalates on the gravities were approximately linear functions of the amounts added. The results, in terms of the effect per mg. of oxalate added per ml. of whole blood, are given in Table VIII.

TABLE VIII

Changes in Gravity of Whole Blood and Plasma Caused by Addition of Ammonium and Potassium Oxalates

Change measured	Extent of change per mg. oxalate added to 1 ml. blood		
	Ammonium oxalate	Potassium oxalate	Oxalate mixture (3 gm. ammonium oxalate + 2 gm. potassium oxalate)
Plasma gravity	+0.0007	+0.0002	+0.0003
Whole blood gravity	+0.0003	+0.0006	+0.0004
Plasma protein calculated from G_P*			
Gm. per 100 ml. plasma	+0.26	+0.07	+0.11
% of mean normal	+3.5	+1.0	+1.4
Hemoglobin calculated from G_P and G_B†			
Gm. per 100 ml. blood	−0.02	+0.24	+0.10
% of mean normal	−0.1	+1.5	+0.6

* G_P = plasma gravity. For calculation of protein concentration from gravity, see the accompanying paper (9).

† G_B = whole blood gravity. For calculation of hemoglobin from G_B and G_P, see the accompanying paper (10).

The differences, shown in Table VIII, between ammonium and potassium oxalates with regard to their relative effects on plasma and whole blood gravities, respectively, are presumably attributable to differences in NH_4^+ and K^+ with regard to penetration into the cells. The ammonium-potassium oxalate mixture of Heller and Paul is chosen for routine use because its effects are about equal on plasma and whole blood gravities and on the plasma protein and hemoglobin values calculated therefrom.

SUMMARY

A simple technique is described for measuring the specific gravity of plasma, serum, or whole blood. The method is based on the facts that a drop of plasma or blood falling into a solution of copper sulfate of similar gravity is instantly encased in a sac of copper proteinate, and that the drop remains discrete and without change in gravity for about 10 seconds. During this interval the rise or fall of the drop indicates the relation of its gravity to that of the copper sulfate solution.

3 or 4 drops of blood or plasma and about a minute of time suffice for a gravity measurement. No temperature controls or corrections are needed, because the temperature coefficients of blood and plasma are so near those of the corresponding copper sulfate solutions that temperature changes between 10° and 40° do not significantly affect the gravity relations.

Drops of whole blood were found to balance in copper sulfate solutions of the same gravity. The standard deviation of blood gravity determined by the copper sulfate method was ±0.0004 gravity unit from the blood gravity measured by pycnometer.

Drops of human plasma were found to balance in copper sulfate solutions averaging 0.0007 gravity unit lighter than the plasma. The standard copper sulfate solutions used for plasma are prepared to correct for this difference. The standard deviation of plasma gravity determined by the copper sulfate method was ±0.0003 gravity unit from the plasma gravity measured by pycnometer.

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THE ESTIMATION OF PLASMA PROTEIN CONCENTRATION FROM PLASMA SPECIFIC GRAVITY

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In 1930 Moore and Van Slyke (1) reviewed the various physical measurements, refractometric and others, that might serve as measures of the protein concentration of plasma, and concluded that specific gravity was the most reliable if application was required to pathological as well as normal plasmas. For plasma of nephritic patients they found that the relation between protein concentration and specific gravity, D_{20}^{20}, was expressed by the linear equation, $P = 343\ (G - 1.0070)$, P representing protein concentration in gm. per 100 ml. and G the specific gravity, D_{20}^{20}. The constants 343 and 1.0070 were based on gravity measurements by pycnometer and on proteins calculated from Kjeldahl nitrogen as 6.25 × N.

A number of authors have since published modifications of the equation $P = a(G - b)$, with differing constants based on gravities measured by several procedures, and on micro-Kjeldahl analyses also by various procedures. The procedures and the constants arrived at for the equation are indicated in Table I. The gravity method indicated as $CuSO_4$ is the procedure described in the preceding paper (2), but without the correction of 0.0007 for the difference between D_{25}^{25} of copper sulfate standards and the plasma drops that balance in them. The gradient column method is that of Linderstrøm-Lang in the modification described by Lowry and Hunter (3). The Barbour method is the "falling drop" method described by Barbour and Hamilton (4). In order to compare calculations by the different equations the protein concentration calculated by each equation for plasma which has a gravity of 1.0270 determined by pycnometer is indicated in the column "P calculated."

From a recently published experimental review of Kjeldahl procedures (13) it appears probable that the differences in the relation between specific gravity and Kjeldahl-determined protein nitrogen found by different investigators (last column of figures, Table I) may in part be due to the fact

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TABLE I

Variations of Equation $P = a(G - b)$ Formulated by Different Investigators

Authors and plasma donors	Year	Specific gravity method used	Values of a and b used		Value of P calculated for plasma of G = 1.0270 by pycnometer		Method used to determine proteins
			a	b	G by method used	P calculated	
					D_t^t	*gm. per 100 ml.*	
Moore and Van Slyke (1)	1930						Micro-Kjeldahl, digested with H_3PO_4, H_2SO_4, $K_2S_2O_8$ until clear
9 normal men		Pycnometer	358	1.0070	1.0270	7.16	
Nephritic patients		"	343	1.0070	1.0270	6.86	
Weech, Reeves, and Goettsch (5); dogs, mostly hypoproteinemic	1936	"	340	1.0069	1.0270	6.83	Micro-Kjeldahl, no details
Cole, Allison, and Boyden (6); rabbits, normal	1943	$CuSO_4$	325	1.0064	1.0263*	6.47	" "
Hoch and Marrack (7, 8); human, normal	1945	Pycnometer	366	1.0070	1.0270	7.30	Micro-Kjeldahl, digested 1–3 hrs. with Cu and Se catalysts
		Gradient column	361	1.0070	1.0271†	7.25	
		$CuSO_4$	364	1.0060	1.0260†	7.30	
Lowry and Hunter (3); human	1945	Gradient column	348	1.0069	1.0275†	7.08	Micro-Kjeldahl, no details
Lloyd *et al.* (9); human, normal	1945	" "	301	1.0027	1.0270†	7.32	Gravimetric
Meyer *et al.* (10); dogs	1945	Barbour	315	1.0050	1.0263	6.41	Micro-Kjeldahl, Cu and Se catalysts; digested 10 min.
		$CuSO_4$	325	1.0059	1.0263*	6.63	
Dicker (11); rats	1948	"	364	1.0060	1.0263*	7.39	Micro-Kjeldahl, Cu and Se catalysts; digested 5 hrs.
Lloyd *et al.* (12); human with famine edema	1948	Gradient column	354	1.0073	1.0270†	6.97	Gravimetric

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* The authors quoted, following the usage of a preliminary report by the present writers, took the D_t^t of plasma as equal to the D_t^t of the $CuSO_4$ solution in which the plasma drop balanced. As shown in the preceding paper (2), by this procedure plasma D_t^t is estimated on the average as 1.0263 when the pycnometer value is 1.0270. Meyer *et al.* (10) in their measurements found the same gravities by the Barbour falling drop method as by the preliminary copper sulfate procedure; hence the same correction of 0.0007 is applied to their gravity measurement by the Barbour method.

† G values by methods other than pycnometer, as found for plasmas with pycnometer G of 1.0270, by the authors cited.

that most of the Kjeldahl digestion procedures used give low results when applied to plasma protein. Hiller *et al.* (13) found that nitrogen values equal to those yielded by Dumas combustion were obtained only when mercury and potassium sulfate were used as catalysts, or when digestion with copper and selenium as catalysts was prolonged for several hours. It will be noted in Table I that, for plasma of $D_t^t = 1.0270$, the protein concentrations estimated by Hoch and Marrack and by Dicker, who digested for 1 to 5 hours, and by Lloyd *et al.* (9), who determined the proteins gravimetrically, are higher than the concentrations estimated by authors who used shorter or unstated Kjeldahl digestions.

The work presented in this paper was carried out to ascertain with as much accuracy as possible the relation of plasma specific gravity to human plasma protein concentration calculated from nitrogen analyses.

Methods

Blood for the analyses was drawn and treated with heparin as described in the preceding paper (2). For the data on normal subjects in Table III blood samples of about 20 ml. were drawn; 10 ml. of the plasma were used for determination of gravity by pycnometer as described in the preceding paper (2). Portions were then used for check determinations of the gravity by the copper sulfate method (2), and for macro-Kjeldahl nitrogen determinations. For the data on hospital patients reported in Table II, portions of 5 ml. or less of blood were drawn, and gravities were determined by the copper sulfate method (2). In the determinations by the copper sulfate method, the gravity of the plasma is taken as 0.0007 unit above the gravity of the copper sulfate solution in which the plasma drops balance. (This correction is automatically made when the standard copper sulfate solutions are prepared according to Table II of the preceding paper (2).)

The methods used for total nitrogen determination are indicated in the headings of Tables II and III. The method of Hiller *et al.* (13), with Hg and K_2SO_4 as catalysts in the digestion, was found (13) to give nitrogen values for proteins equal to those obtained by Dumas combustion. The analyses by the Campbell-Hanna (14) method, with Cu and Se as catalysts, were performed before the more accurate procedure of Hiller *et al.* had been worked out. The Campbell-Hanna method was modified by prolonging the digestion for 2 hours after the digest cleared. In a series of analyses by both methods, the Campbell-Hanna procedure was found to yield 98.4 per cent as much nitrogen as the procedure of Hiller *et al.* (13) with mercury catalyst; hence nitrogen values obtained by the Campbell-Hanna method were multiplied by the correction factor 1.016.

Protein nitrogen was calculated by subtracting non-protein nitrogen (trichloroacetic acid filtrate N determined by gasometric micro-Kjeldahl (15)) from the total plasma nitrogen. The weight of protein was calculated as 6.25 × N, the factor 6.25 being that obtained by Hiller *et al.* (13) in analyses of fat- and ash-free preparations of normal human plasma protein.

TABLE II

Plasmas from Hospital Patients

G values all determined by the copper sulfate method (2).

	Miscellaneous hospital patients	Nephritic patients
No. of plasmas*	50	41
Type of Kjeldahl analysis used to determine P_N	Macro, with Cu and Se catalysts, 2 hrs. digestion (14)†	Micro, with Hg and K_2SO_4 catalysts (13)
Range of P_N, *gm. per 100 ml.*	5.62-12.44	3.71-8.03
Mean P_N, *gm. per 100 ml.*	7.282	4.732
“ G	1.02697	1.01996
“ value of b in equation $P_N = 365 (G - b)$	1.00702	1.00700
	gm. per 100 ml.	*gm. per 100 ml.*
Comparison of P_G calculated as $365 (G - 1.0070)$ with P_N calculated as 6.25 × N		
Mean algebraic difference, $P_G - P_N$	+0.014	±0.000
Standard deviation of P_G from P_N	±0.22	±0.26
Maximal + “ “ “ “ “	+0.58	+0.53
“ − “ “ “ “ “	−0.32	−0.52

* Plasmas with non-protein nitrogen over 100 mg. per 100 ml. are excluded from this table, and reported in Table V. In some cases two or more plasmas were drawn from the same patient on different dates.

† The nitrogen values obtained by the digestion with Campbell and Hanna's (14) mixture of H_2SO_4, H_3PO_4, Cu, and Se were multiplied by the factor 1.016, to correct for the fact that this digestion was found to give 1.6 per cent less ammonia than the digestion with Hg and K_2SO_4 (13).

Symbols

The following symbols are used. P = gm. of protein per 100 ml. of plasma. G = specific gravity of plasma in terms of D^{25}_{25}, the density of water at the same temperature as that of the plasma being taken as unity ($0.997G$ = the density D^{25}_{4}). When it is desirable to distinguish between proteins calculated from nitrogen determinations and proteins calculated from plasma specific gravities, P_N is used to indicate the former and P_G the latter. V_P indicates the "apparent specific volume" of the proteins in

TABLE III

Plasmas of Normal Adults

Range of normal specific gravity and protein concentration. Constants of equation, $P = a(G - b)$. Comparison of protein concentration, P_N, calculated from Kjeldahl nitrogen, with concentration, P_G, calculated from plasma gravity.

	Group I, 17 subjects		Group II, 20 subjects
Type of Kjeldahl analysis used to determine P_N	Macro, with Hg and K_2SO_4 catalysts, 2 hrs. digestion (13); samples of 2 ml. plasma		Macro, with Cu and Se (14), 2 hrs. digestion*
Range of normal P_N calculated as 6.25 × protein N			
Mean, *gm. per 100 ml.*	7.39		7.38
s.d. from mean, *gm. per 100 ml.*	±0.34		±0.28
Maximum observed, *gm. per 100 ml.*	8.03		8.17
Minimum " " " *100* "	6.76		7.05
	G by pycnometer	G by $CuSO_4$ method	G by $CuSO_4$ method
Range of normal gravity			
Mean G	1.02683	1.02681	1.02678
s.d. from mean	±0.0099	±0.0091	±0.0086
Maximum observed G	1.0287	1.0283	1.0285
Minimum " "	1.0247	1.0249	1.0252
Mean value of a calculated as $a = \frac{P_N}{(G - 1.0070)}$	372.7	373.0	373.1
	gm. per 100 ml.	*gm. per 100 ml.*	*gm. per 100 ml.*
Comparison of P_G calculated as 373 $(G - 1.0070)$ with P_N calculated as 6.25 × N			
Mean algebraic difference, $P_G - P_N$	+0.007	±0.000	−0.002
s.d. of P_G from P_N	±0.093	±0.106	±0.136
Maximal + deviation of P_G from P_N	+0.14	+0.15	+0.17
" − " " " " "	−0.15	−0.25	−0.31

* See foot-note (†), Table II.

plasma, defined as the volume of solvent (water + non-protein solutes) that is displaced by 1 gm. of protein.[1]

Estimation of Constants of Equation, $P = a(G - b)$

The range of protein concentrations in normal plasmas is too narrow to indicate closely the best values for the constants. Thus a and b respectively may be taken for normal plasma as 360 and 1.0063, 370 and 1.0069, or 380 and 1.0074, without a statistically significant difference in the accuracy of the equation. Consequently we have used the wider protein concentration range of pathological plasmas to estimate the best line (Fig. 1) which is defined by Equation 1 (Table IV).

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[1] For a discussion of "apparent partial specific volumes" of proteins in solution, see Kraemer (16).

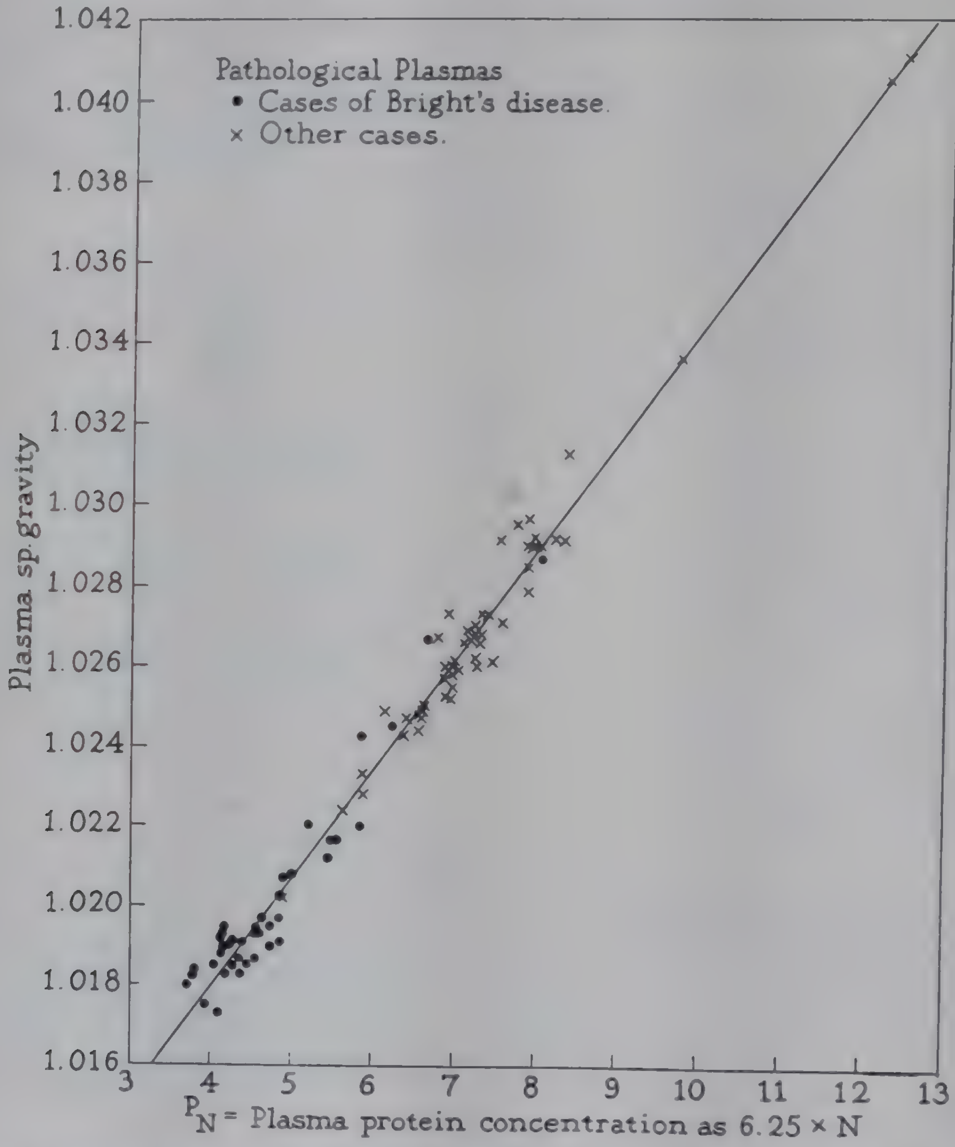

FIG. 1. Relation of specific gravity to nitrogen-calculated protein in pathological plasmas.

When this equation was used to calculate proteins from gravities for the normal plasmas of Table III, the resulting P_G values averaged 2.2 per cent lower than the P_N values. The difference is indicated by the greater a value of Equation 2 (Tables III and IV), b being kept constant at 1.0070.

Equation 3 (Table IV) is used for computing the nomograms for general use in the following paper (17). Equation 3 gives on the average plasma protein concentrations 1 per cent lower than nitrogen-calculated values of the normal plasmas of Table III, and 1 per cent higher than the nitrogen-calculated values of the pathological plasmas of Table II.

Factors Determining Constant a in Equation $P = a(G - b)$—From the equation it is evident that $1/a$ represents the increase in gravity per increase of 1 gm. per 100 ml. in protein concentration. An a of 373 indicates that an increase of 1 gm. per 100 ml. in P causes an increase of $1/373 = 0.00268$ in G, owing to replacement of solvent (water + non-protein solutes) of gravity b by protein of greater density.

The quantitative relation of a to the specific volume of the dissolved proteins may be calculated as follows: Increase of 1 gm. per 100 ml. in P displaces V_P ml. of solvent of density $0.997b$. The weight of solvent displaced is $0.997bV_P$ (density × volume). The resultant increment of weight per 100 ml. of plasma is $1 - 0.997bV_P$, and the increment in weight per 1 ml., or in D_4^{25}, is $(1 - 0.997bV_P)/100$. Since $D_4^{25} = 0.997D_{25}^{25} = 0.997G$, the increment in G caused by an increase of 1 gm. in P is $(1 - 0.997bV_P)/99.7$. This increment is $1/a$. Hence

TABLE IV

Equations for Calculating Plasma Protein Concentration, P, from Specific Gravity, G

Equation No.	Type of plasma	Formula to calculate protein, P, from gravity, G
1	Pathological	$P = 365(G - 1.0070)$
2	Normal	" $= 373($" $- 1.0070)$
3	" and pathological	" $= 369($" $- 1.0070)$

$$a = \frac{99.7}{1 - 0.997bV_P} \tag{4}$$

Rearranging to calculate V_P gives

$$V_P = \frac{a - 99.7}{0.997ab} \tag{5}$$

From the a and b values of Equation 2, for normal plasmas, one calculates $V_P = 0.730$. Hence an increase of 1 gm. per 100 ml. in P involves the displacement of 0.730 ml., or $0.730 \times 1.0070 \times 0.997 = 0.7329$ gm., of solvent, and an increase of $1 - 0.7329 = 0.2671$ gm. in the weight of 100 ml. of plasma. There results an increase of 0.002671 in the density D_4^{25}, and an increase of $0.002671/0.997 = 0.002679$ in D_{25}^{25}.

From observations of Adair and Adair (18), it appears that proteins, unlike most crystalloid solutes, may dissolve without measurable shrinkage in volume, the volume of the solution formed equaling the volume of the solvent plus the volume of the dry protein. In the case of edestin and hemoglobin the apparent specific volumes of both proteins were found to be 0.744 ± 0.002 in aqueous solution, and the specific volume (1/(density)) in the dry state was also found to be 0.744 ± 0.002, from density values of 1.345 ± 0.003. The apparent behavior of the proteins in dissolving without volume change is consistent with the linear form of the equation, $P = a(G - b)$.

From the a of 365 of Equation 1, one calculates for the pathological plasmas of Table II a V_P of 0.724, smaller than the V_P of 0.730 calculated for normal plasma. Pedersen (19) found for human albumin a V_P of 0.736, for γ-globulin, 0.718. It consequently appears possible that changes in the ratio of γ-globulin to albumin could cause changes in V_P of the order required to cause the average difference of 2 per cent in P_N between the normal and pathological plasmas of the same gravity in our series.

For a protein, fetuin, from the plasma of a new-born calf, Pedersen (19) found a V_P of 0.70, suggesting that the presence of abnormal proteins, as well as changes in the distribution of normal ones, may influence V_P and hence the a value of the equation $P = a(G - b)$.

Fig. 2 shows no correlation between the A:G ratio, determined by the Howe method, and the $P_G - P_N$ difference. Present data do not suffice to show whether correlation would become evident if the γ-globulin fraction was determined separately, and if the influence of other factors, such as error in calculating protein as 6.25 × N in pathological plasma, were eliminated.

Factors Determining Constant b in Equation $P = a(G - b)$—The value 1.0070 of the constant b approximates the gravity of a solution of the plasma crystalloids. This was demonstrated by an experiment in which normal plasma was filtered under pressure through cellophane. Filtrates, free of protein, showed by micro pycnometer D_{25}^{25} values approximating 1.0070 within the limit of error, ±0.0003, of the measurement.

Per unit concentration, the increment in gravity caused by the organic crystalloids is of the order of the increment caused by protein (0.0027), while the mineral constituents cause from 2 to 2.5 times as great increments. Per gm. per 100 ml., the increments caused by the chief plasma crystalloids are as follows: urea 0.0029, glucose 0.0039, $NaCl$ 0.0071, $NaHCO_3$ 0.0062, KCl 0.0064, $CaCl_2$ 0.0087. The total increment in gravity due to the crystalloid solutes of normal plasma may be approximately estimated by assuming that of the total mineral salts, indicated by a total base of 154 m.eq. per liter, 105 m.eq. have the effect of $NaCl$ and the remainder the effect of $NaHCO_3$, and that the effects of the nitrogenous crystalloids approximate that calculated by assuming that all the non-protein nitrogen is urea. The respective gravity increments caused by the solutes thus calculated are NaCl 0.0036, other mineral salts 0.0025, glucose (100 mg. per 100 ml.) 0.0004, non-protein nitrogenous solutes (30 mg. of non-protein N per 100 ml.) 0.0002, total increment 0.0067.

The lipides, as non-protein constituents, could affect the value of b if they altered significantly the gravity of the non-protein phase of the plasma. From the fact that fats in general are lighter than water, one might expect their presence in plasma to lower the gravity of the non-protein phase, and to cause markedly negative errors in gravity-calculated proteins when the plasma lipides are high. However, Popjak and McCarthy (20) found that the mean apparent partial specific volume of the lipides in plasmas examined by them was 0.996, indicating a value of 1.004 for the density, D_4^{25}, of the lipide phase; this corresponds to a D_{25}^{25} of 1.0070, identical with the b value, assumed in Table IV as the D_{25}^{25} of the non-protein phase of plasma. In part, the apparent high density of the lipide phase, compared with ordinary fat, is attributable to the fact that about 40 per cent of the mixed plasma lipides ordinarily consists of cholesterol, which has a density of 1.067. It is possible also that the loose combination of the lipides with proteins, which makes varying amounts of lipides invisible in plasma, may

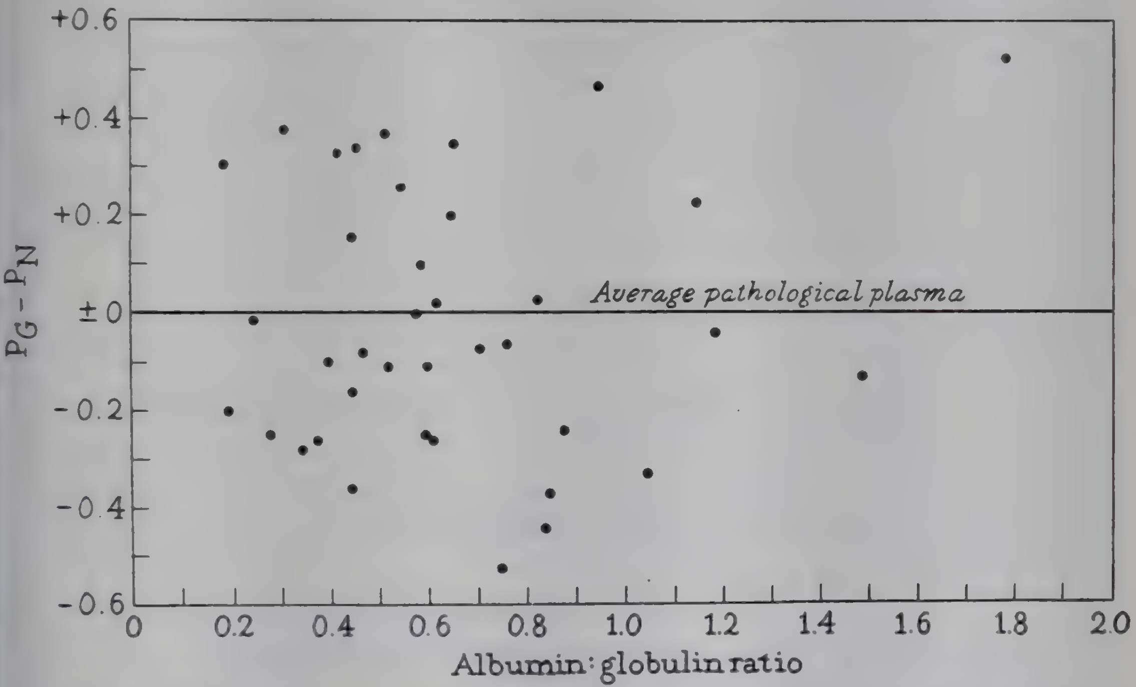

Fig. 2. Lack of correlation between the albumin-globulin ratio and the $P_G - P_N$ difference in nephrotic plasmas.

decrease the apparent specific volume of the lipides. The plasma lipides, at least up to the maximal concentrations (about 3 gm. per 100 ml.) studied by Popjak and McCarthy (20), appear to have little effect on the relation of plasma gravity to protein concentration.

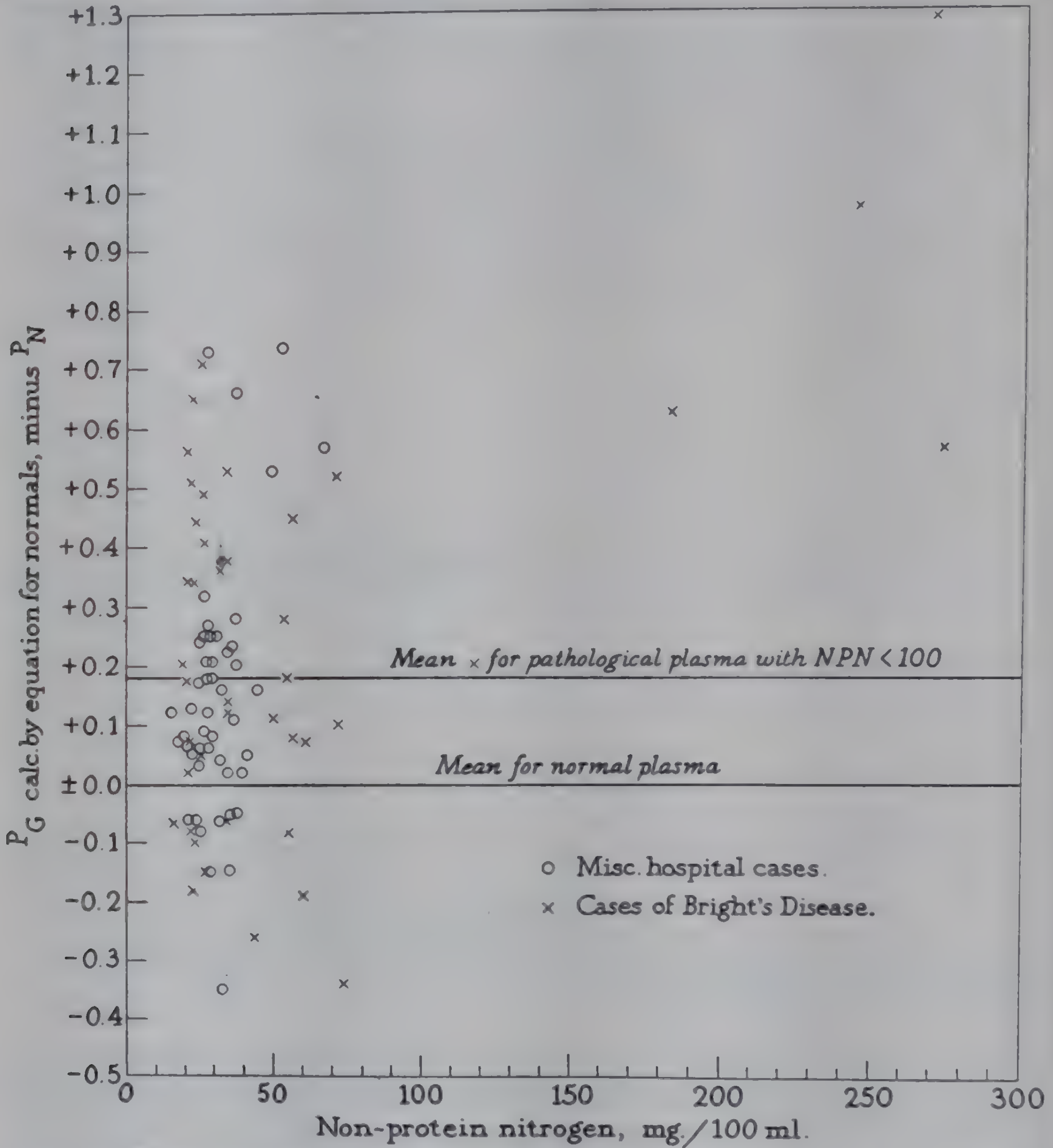

FIG. 3. Effect of high plasma non-protein nitrogen concentrations on the $P_G - P_N$ differences in pathological plasmas.

In Table II a large proportion of the subjects in the group with Bright's disease were lipemic, but this group, like the miscellaneous pathological group in the same table, showed P_G values that, if calculated by the equation for normal plasma, averaged 2 per cent *above* the nitrogen-calculated proteins (see Figs. 2 and 3).

In exceptional plasma, with lipemia so gross that a large layer of fat could be removed by centrifugation, an increase in plasma gravity as the result of centrifugation has been observed of an order to indicate that the material removed had a D_{25}^{25} less than 1.007; but even in such cases, some of which are included in Fig. 2, the effect of the lipides on P_G did not lower the latter by more than 0.2 gm. per liter.

Sources of Error in Calculation of Weights of Proteins As 6.25 × N in Pathological Plasma

Part of the deviation of P_G from P_N noted in Table II is probably due to error, not in P_G, but in P_N calculated as 6.25 × N. The factor, 6.25, is based on an assumed constant protein nitrogen content of 16.0 per cent, found in analyses of fat- and ash-free total proteins of normal human plasma (13). The different fractions of normal human plasma, analyzed by Dumas combustion by Brand, Kassell, and Saidel (21), had the following percentages of nitrogen: albumin 15.95, β-globulin 14.84, γ-globulin 16.03, fibrinogen 16.90. It is evident that either abnormalities in the proportions in which the different normal fractions are present in the total plasma protein mixture, or the presence of abnormal proteins, may significantly alter the percentage of nitrogen in the mixture, and the accuracy of the calculation of gm. of protein as 6.25 × gm. of N. Elimination of variation in protein nitrogen content as a source of error in chemically determined proteins could be accomplished by determining the proteins gravimetrically (22), as has been done by Lloyd *et al.* (12) in cases of famine edema, or by wet carbon combustion of the lipide-free proteins by the method of Hoagland and Fischer (23).

A relatively small plus error is introduced into P_N by the nitrogen content of the phosphatides. The aqueous protein precipitants ordinarily used, such as trichloroacetic and tungstic acid solutions, form protein precipitates which carry down with them all of the lipides of plasma. Hence in the usual procedures for determining protein nitrogen, including that of the present papers, the nitrogen of the phosphatides is included. The approximate effect may be estimated as follows: The phosphatides comprise about 25 per cent of the total lipides. The nitrogen content of the phosphatides is about 2 per cent; hence the nitrogen content of the total lipides is about 0.5 per cent. An increase in plasma lipides of 3 gm. per 100 ml. would increase the apparent P_N by about 0.1 gm. per 100 ml., and make the gravity-calculated proteins appear by comparison to be that much too low. The error in P_N caused by lipide nitrogen is negligible (about 0.02 gm. per 100 ml.) in normal plasma, but may become significant in gross lipemia. Such an effect could be a factor in some of the relatively few cases in Figs. 2 and 3 in which P_G calculated by the equation for normal plasma falls below P_N.

Magnitude of Intrinsic Error in Calculation of Proteins from Gravity in Normal and Pathological Plasmas

By intrinsic error is meant error in excess of that due to error in measurement of the specific gravity; it is the error due to variation of a and b in the equation $P = a(G - b)$ from their assumed values. To estimate the magnitude of the maximal intrinsic error from results in Tables II and III, we subtract from the observed maximal $P_G - P_N$ difference the sum of the estimated effects of maximal errors in the gravity and the Kjeldahl methods used, and obtain a result which is presumably larger than the true intrinsic error of P_G (because error in P_N due to variation from the factor 6.25 is ignored), but is the best estimate that can be made at present of the intrinsic error.

As maximal experimental errors in Tables II and III we will assume ±0.0001 for gravities measured by pycnometer, ±0.0006 for gravities measured by the copper sulfate method (2), and ±0.5 per cent of the nitrogen determined by Kjeldahl analysis (13). The gravity errors of the two methods correspond to errors of ±0.04 and ±0.22 gm. respectively of protein per 100 ml. of plasma. The nitrogen error, for a plasma of mean normal protein content, corresponds to ±0.04 gm. of protein per 100 ml. The combined maximal experimental errors of P_G and P_N thus estimated are ±0.08 gm. of protein per 100 ml. of plasma when gravities are measured by pycnometer and ±0.26 gm. when gravities are measured by the copper sulfate method. Errors in P_N that may be due to deviations from the factor 6.25 are not included in the estimates.

Normal Plasma—In the three groups of observations in Table III, the maximal deviations of P_G from P_N, calculated as twice the standard deviations, are ±0.19, ±0.21, and ±0.27 gm. per 100 ml., compared with estimated maximal experimental errors of ±0.08, ±0.26, and ±0.26 gm. per 100 ml., respectively. The observed standard deviation × 2 exceeds the estimated experimental errors by 0.11 gm. per 100 ml. in the first group, falls short by 0.05 in the second, exceeds by 0.01 in the third.

It appears probable that the intrinsic error in the calculation of protein concentrations in normal human plasma from gravities by Equation 2 does not exceed 0.1 gm. per 100 ml. It also appears that deviations of the protein nitrogen content from 16.0 per cent in normal plasma are small; otherwise error in P_N from such variation would have caused the deviation of P_G from P_N to exceed more definitely the deviation predictable from the errors in gravity measurement and Kjeldahl analysis.

Pathological Plasmas—In the pathological plasmas of Table II, the maximal deviations of P_G from P_N, calculated as twice the standard deviations, are 0.44 and 0.52 gm. per 100 ml. in the two groups. These definitely exceed the 0.26 gm. difference estimated as the maximum attributable to errors in the Kjeldahl nitrogens and in the gravities determined by the copper sulfate method. The intrinsic error of gravity-estimated proteins in pathological plasmas of the types covered by the cases in Table II would accordingly be estimated at about 0.2 gm. per 100 ml., if the P_N values could be assumed to be without error from use of the factor 6.25. Since there is reason to assume that part of the deviation of P_G from P_N in pathological plasmas may be due to error in the factor 6.25, it appears probable that the maximal intrinsic error of gravity-calculated proteins in pathological plasmas of the type in Table II (without non-protein N over 100 mg. or very high blood sugar) is something less than 0.2 gm. per 100 ml.

More extensive data, with all gravities precise to ±0.0001 unit, and with chemical determinations of the proteins by a method not dependent on an assumed constant nitrogen content, would provide more satisfactory statistics for estimation of the intrinsic error of P_G. The above estimates, however, appear to provide a fair approximation of the accuracy with which proteins can be calculated in human plasma from gravities measured without significant error.

Probable Causes of Greater Deviations of Gravity-Calculated from Nitrogen-Calculated Proteins in Pathological Than in Normal Plasmas—In cases of gross glucemia, or such high blood non-protein nitrogen concentrations as are exemplified in Table V, abnormally high crystalloid concentrations can cause gravity-calculated proteins to be too high.

If cases with such gross crystalloid increases (Table II) are excluded, however, pathological plasmas show a variability in the relation of gravity-calculated to nitrogen-calculated proteins that appears to be due to variations in either the nitrogen contents or specific volumes of the proteins themselves. The fact that, for a given specific gravity, the average plasma protein content was 2 per cent less in the pathological plasmas of Table II than in the normal plasmas of Table I could be due either to lower nitrogen content (15.7 *versus* 16.0 per cent) or lower specific volume (0.724 *versus* 0.730) of the proteins of the pathological group.

Some of the results in the literature appear to indicate also that, in conditions of malnutrition, abnormalities in the plasma proteins similarly affect the $P_G - P_N$ difference. Hoch and Marrack (8) observed that, in plasmas from a group of subjects suffering from malnutrition, the nitrogen-calculated protein content averaged 0.54 gm. per 100 ml. lower than in normal plasmas of the same specific gravity, the abnormality being in the same direction observed in the pathological plasmas of Table II. Because there is no reason to suspect the presence of such gross increases of crystalloid solutes (*e.g.* 400 mg. of glucose or 500 mg. of urea per 100 ml. above normal) as would be necessary to cause the observed effect in Hoch and Marrack's subjects, it appears that the observed $P_G - P_N$ difference may have been due to abnormality in the proteins. The abnormal relation between P_N and P_G in the malnourished group could be caused either by a decrease in specific volume of the proteins from the 0.730 of normal plasma proteins to 0.706 in the malnourished group, or by a decrease in nitrogen content from the 16 per cent in normal proteins to 14.8 per cent in the malnourished group, or by smaller shifts in both values.

TABLE V

Cases with Non-Protein Nitrogen Greater Than 100

Case No.	G by $CuSO_4$ method (*a*)	$P_G = 365 \times (G - 1.0070)$ (*b*)	$P_N = 6.25 \times$ protein N (*c*)	Difference, $P_G - P_N$ (*d*)	Non-protein N (*e*)	Error of P_G estimated by assuming excess non-protein N* is urea (*f*)
		gm. per 100 ml.	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*
1	1.0280	7.67	6.63	+1.04	0.272	+0.55
2	1.0242	6.28	5.58	+0.70	0.249	+0.50
3	1.0256	6.79	6.48	+0.31	0.275	+0.55
4	1.0249	6.53	6.17	+0.36	0.185	+0.35

* Excess non-protein nitrogen is taken as the amount above 0.030 gm. per 100 ml. The increment of G per gm. of urea per 100 ml. is taken as 0.0029 (see p. 338), and hence as 0.00621 per gm. of urea N per 100 ml. The effect of "excess non-protein N" on G is hence estimated as a G increment of 0.00621 × "excess non-protein N," and the error in P_G caused by non-protein N is taken as 365(0.00621 × "excess non-protein N") = 2.27 × "excess non-protein N."

A similar difference from normal subjects (9) in subjects with famine edema (12) was noted by Lloyd *et al.* (see Table I). Lloyd *et al.* compared the gravities of the plasmas of their famine group with protein concentrations determined, both gravimetrically, as described by Robinson and Hogden (22), and by Kjeldahl, and concluded that both factors, low protein specific volume and low protein nitrogen content, contributed to the positive value of the $P_G - P_N$ difference. Gravimetrically determined protein concentrations averaged 0.35 gm. per 100 ml. lower in plasma of the same gravity in the famine-stricken than in the normal group (compare data of Lloyd *et al.* for 1945 and 1948, Table I); this difference, being independent of nitrogen values, may be attributable to smaller specific volume (greater density) of the proteins in the starved group. If this is the case, the equation $P = a(G - b)$ would require, for starved subjects, an a value still smaller than that of Equation 1 (Table IV).

Apparent Partial Specific Volume of Proteins in Normal Human Plasma[1]

The data of Table III permit calculation by Equation 5 of the partial specific volume of the proteins in normal plasma from the a and b values of the equation $P = a(G - b)$. As pointed out, the a and b values may be varied considerably without a statistically significant change in the accuracy of the equation for calculating P. However, it appears that the correct value for a in normal plasma, with G measured by pycnometer, is within the range 373 ± 15, with the corresponding b in the range 1.0070 ± 0.0009. The corresponding V_P range calculated by Equation 5 from these a and b values is 0.730 ± 0.010. This is not out of line with Pedersen's (19) values of 0.736 for human albumin and 0.718 for γ-globulin, which together constitute about 70 per cent of normal human plasma protein, or with Popjak and McCarthy's (20) value of 0.729 for rabbit proteins. In earlier (1928) work on the proteins of horse serum, Svedberg and Sjögren (24) found higher and nearly identical values, 0.748 for the albumin fraction and 0.745 for the total globulins; however, they determined their protein concentrations by weighing the heat-coagulated proteins, which may have contained some occluded fat.

Range of Normal Plasma Protein Concentration—The data of Table III indicate 7.4 ± 0.6 gm. per 100 ml. as the normal range. The data were obtained by macro-Kjeldahl analyses performed with precautions for accuracy (13), and presumably indicate the approximate range for plasma drawn from the blood of normal healthy young adults in the standing or sitting position. As a number of authors have shown, however (*cf.* Lange (25)), change from the upright to the horizontal position causes a decrease in plasma protein concentration, which is measurable in a few minutes and approaches a plateau in about an hour. It is due to dilution of the blood by interstitial fluid. Lange found the average protein concentrations in plasma of blood drawn from subjects lying down to be 0.55 gm. per 100 ml. lower than in plasma drawn while the subjects were standing. For subjects in bed, therefore, a range of about 6.8 ± 0.6 gm. per 100 ml. may be normal.

SUMMARY

From comparison of plasma specific gravities with protein concentrations calculated from accurate Kjeldahl analyses as N × 6.25 the following equations have been derived: from thirty-seven normal plasmas, $P = 373\ (G - 1.0070)$; from 91 pathological plasmas without gross elevation of blood urea or sugar, $P = 365\ (G - 1.0070)$. P indicates the gm. of protein per 100 ml. of plasma, G the specific gravity of the plasma, the density of water at the same temperature being taken as unity.

As indicated by the difference between the constants, 373 and 365, the pathological plasmas had on the average 0.98 as much nitrogen-calculated protein per 100 ml. as normal plasmas of the same specific gravity. Data from the literature indicate that plasma of subjects suffering from malnutrition shows a similar difference from normal plasma. The difference could be due to either lower specific volume or lower nitrogen content of the proteins of the abnormal plasmas.

It is estimated that, with errors of gravity measurement eliminated, the error in calculating protein concentration from gravity is less than 0.1 gm. per 100 ml. in normal human plasma, and less than 0.2 gm. in pathological plasma without gross abnormalities of crystalloid concentration, such as sugar or urea concentrations above 200 mg. per 100 ml.

The range of nitrogen-calculated protein concentration observed in plasma of blood drawn from healthy men in the upright position was 6.8 to 8.0 gm. per 100 ml., average 7.39.

The apparent specific volume of the proteins in normal human plasma is estimated at 0.730 ± 0.010.

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CALCULATION OF HEMOGLOBIN FROM BLOOD SPECIFIC GRAVITIES

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The specific gravity of the red blood cells (normally about 1.10) so greatly exceeds that of the plasma that the difference between the gravities of whole blood and plasma can serve as a measure of the cell content of the blood. By assuming a constant hemoglobin concentration in the cells, one can estimate also the blood hemoglobin concentration from the gravity values.

The calculation is based on two assumed constants; *viz.*, the specific gravity of the red cells and the hemoglobin concentration of the red cells. Neither of these values is in fact constant. In some abnormal types of blood cells, hemoglobin concentration may fall 20 per cent below normal, with cell specific gravity showing a parallel decrease. However, changes in cell hemoglobin concentration and cell specific gravity parallel each other in such a way that their variations almost cancel each other in their effect on the relation of blood hemoglobin concentration to the concentration calculated from the gravities of whole blood and plasma. In consequence the error, even in grossly abnormal blood, is estimated to be only about 0.15 gm. of hemoglobin per 100 ml. of blood greater than the error attributable to the limit of accuracy of the gravity method used.

Ashworth and Tigertt (1) first formulated the equation for calculating blood hemoglobin concentration from the specific gravities of whole blood and plasma. The present paper reports tests of the accuracy of gravity-calculated hemoglobin concentrations by comparison with hemoglobin values determined by precise gasometric methods in normal and abnormal human blood. The gravity determinations have been made by the copper sulfate method (2). A nomogram (Fig. 1) is presented for rapid calculation of hemoglobin from the whole blood and plasma gravities.

Formulation of Equations for Hemoglobin Calculation

Let D_B, D_C, and D_G be the densities (D_4^t) of whole blood, plasma, and red cells, respectively. Let G_B, G_P, and G_C be the corresponding specific

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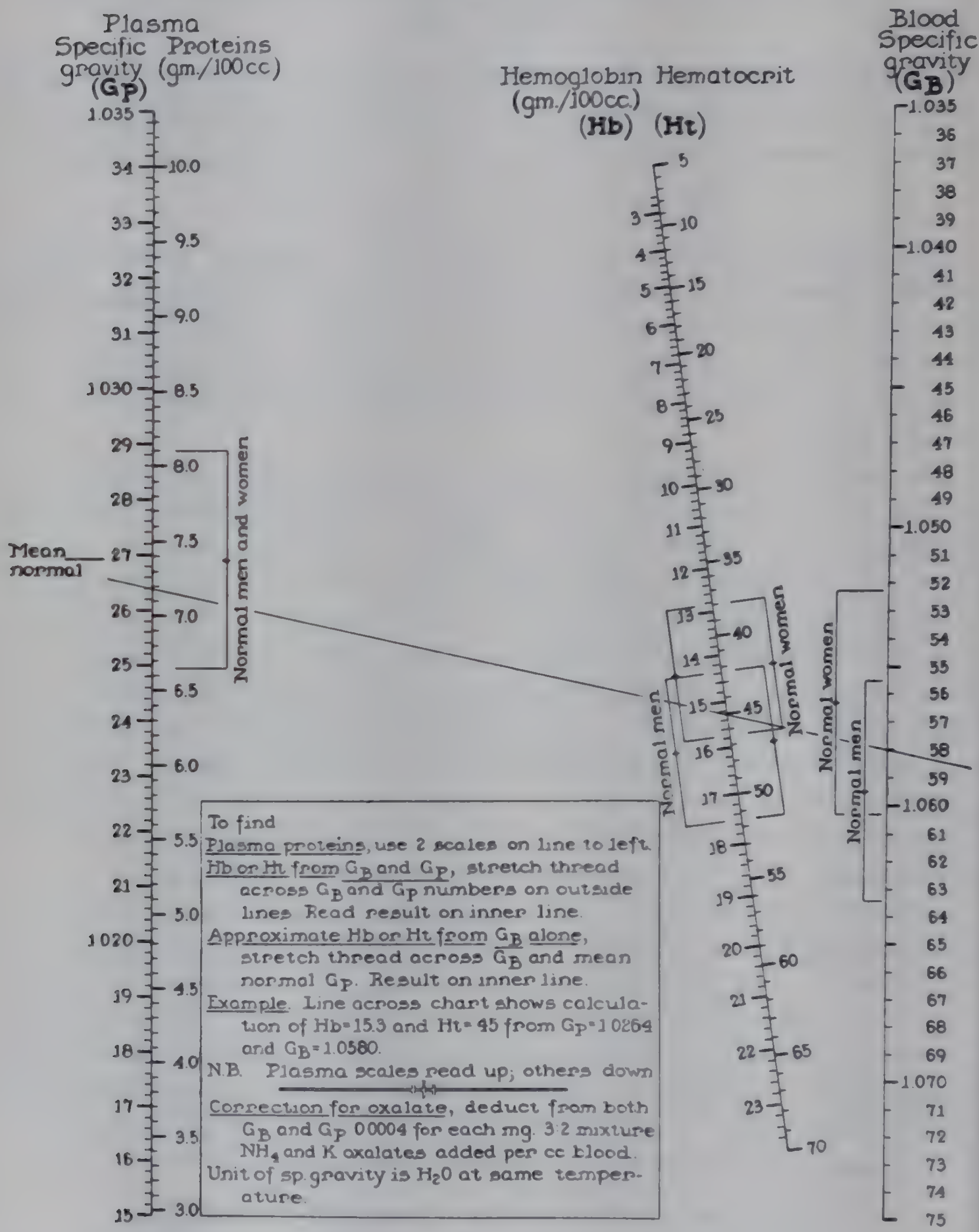
Line chart for calculating plasma proteins, hemoglobin and hematocrit from gravities of plasma and blood



To find

Plasma proteins, use 2 scales on line to left.

Hb or Ht from G_B and G_P, stretch thread across G_B and G_P numbers on outside lines Read result on inner line.

Approximate Hb or Ht from G_B alone, stretch thread across G_B and mean normal G_P. Result on inner line.

Example. Line across chart shows calculation of Hb=15.3 and Ht=45 from G_P=1 0264 and G_B=1.0580.

N.B. Plasma scales read up; others down

Correction for oxalate, deduct from both G_B and G_P 0 0004 for each mg. 3:2 mixture NH_4 and K oxalates added per cc blood.

Unit of sp. gravity is H_2O at same temperature.

FIG. 1.

gravities in terms of D_t^t. Let V_C be the volume of red cells in 1 volume of blood. Let Hb_B be the gm. of hemoglobin in 100 ml. of blood, and Hb_C the hemoglobin in 100 ml. of red cells. The weight in gm. of 1 ml. of blood, which is the density, is the weight of the plasma plus the weight of the cells in 1 ml. of blood.

$$D_B = D_C V_C + D_P(1 - V_C) \tag{1}$$

Since G_B, G_C, and G_P all are the corresponding D values multiplied by the same factor (1.003 when $t = 25°$) the gravity (D_t^t) values may be substituted for densities D_4^t in all terms of Equation 1.

$$G_B = G_C V_C + G_P(1 - V_C) \tag{2}$$

whence

$$V_C = \frac{G_B - G_P}{G_C - G_P} \tag{3}$$

Blood Hb concentration is cell volume × cell Hb concentration.

$$\mathrm{Hb}_B = \mathrm{Hb}_C V_C = \mathrm{Hb}_C \times \frac{G_B - G_P}{G_C - G_P} \tag{4}$$

Constants must be substituted for Hb_C and G_C. Hb_C is calculated as Hb_B/V_C, with Hb_B determined by chemical analysis and V_C by hematocrit under standard conditions. G_C is calculated by rearrangement of Equation 2.

$$G_C = G_P + \frac{G_B - G_P}{V_C} \tag{5}$$

The V_C for Equation 5 is determined by hematocrit.

From data given in the experimental part, Hb_C for normal human blood cells is taken as 33.9 gm. per 100 ml., and the corresponding G_C as 1.0964. We have taken these as the constants. Substituting them for Hb_C and G_C in Equation 4 gives

$$\mathrm{Hb}_B = 33.9 \times \frac{G_B - G_P}{1.0964 - G_P} \tag{6}$$

Cause of Relative Non-Effect of Cell Abnormalities on Calculation of Hemoglobin by Equation 6

As mentioned above, in blood with cells of abnormally low density deviations of Hb_C from 33.9 and of G_C from 1.0964 parallel each other in such a manner that the changes nearly cancel each other, numerator and denominator of the fraction $(33.9(G_B - G_P))/(1.0964 - G_P)$ being changed in nearly the same proportion by parallel deviations of the two constants.

In the hypochromic bloods of Table II, in fact, the correlation between abnormally low G_C and the error in gravity-calculated Hb_B is so slight that it is not statistically definite, although in several bloods G_C is below 1.088 and Hb_C below 29 gm. per 100 ml.

TABLE I

Data from Twenty Normal Men

Subject No.	Directly measured				Calculated			
					Cells		Whole blood	
	G_B, whole blood gravity	G_P, plasma gravity	Hematocrit, centrifuged cells per 100 ml. blood	HbO_2, blood Hb by O_2 capacity	G_C by Equation 5	$\text{Cell Hb} = \frac{100\ \text{HbO}_2}{\text{Hematocrit}}$	Hb from G_B and G_P	
							By Equation 6	Deviation from HbO_2
	D_{25}^{25}	D_{25}^{25}	*ml.*	*gm. per 100 ml.*	D_{25}^{25}	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*
1	1.0635	1.0275	51.2	17.56	1.0978	34.3	17.66	+0.10
2	625	262	48.0	17.46	1018	36.4	17.53	+0.07
3	618	265	49.0	17.10	985	35.6	17.12	+0.02
4	605	277	48.4	16.46	955	34.0	16.18	−0.28
5	615	287	48.0	16.35	970	34.0	16.42	+0.07
6	602	275	47.0	15.99	971	34.0	16.09	+0.10
7	592	265	48.0	15.99	946	33.3	15.86	−0.13
8	585	263	46.2	15.92	960	34.5	15.58	−0.34
9	595	270	48.0	15.84	949	33.0	15.88	+0.04
10	597	285	47.0	15.82	949	33.7	15.58	−0.24
11	590	265	46.0	15.78	971	34.3	15.76	−0.02
12	588	263	47.3	15.68	950	33.2	15.72	+0.04
13	595	277	48.0	15.63	939	32.5	15.69	+0.06
14	582	267	46.0	15.47	952	33.6	15.32	−0.15
15	588	265	46.4	15.46	961	33.3	15.66	+0.20
16	587	277	45.0	15.12	966	33.6	15.30	+0.18
17	582	273	44.0	15.12	976	34.3	15.16	+0.04
18	568	250	44.3	15.11	968	34.1	15.10	−0.01
19	570	257	45.0	15.03	952	33.4	15.01	−0.02
20	577	267	45.0	15.01	956	33.3	15.08	+0.07
Mean	1.0595	1.0269	46.9	15.90	1.0964	33.9	15.89	−0.01
S.D. from mean	±0.0021	±0.0009	±1.8	±1.00	±0.0018			±0.15
Maximum + deviation from HbO_2								+0.20
Maximum minus deviation from HbO_2								−0.34

Estimation of Hemoglobin from Specific Gravity of Whole Blood Alone

In blood with plasma gravities within the normal range 1.027 ± 0.002 (Table I) one can, without much increase in error, estimate Hb_B from G_B

TABLE II

Data from Women Rejected As Blood Donors, Apparently Healthy But with Subnormal Hemoglobin

	Directly measured				Calculated			
					Cells		Whole blood	
							Hb from G_B and G_P	
Subject No.	G_B, blood specific gravity	G_P, plasma specific gravity	Hematocrit, centrifuged cells per 100 ml. blood	HbCO, blood Hb by CO capacity	G_C by Equation 5	Cell Hb = $\frac{100\ \text{HbCO}}{\text{Hematocrit}}$	By Equation 6	Deviation from HbCO
	D_{25}^{25}	D_{25}^{25}	*ml.*	*gm. per 100 ml.*	D_{25}^{25}	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*
1	1.0525	1.0255	39.9	12.96	1.0932	32.5	12.91	−0.05
2	510	265	37.6	11.65	917	31.0	11.88	+0.23
3	506	275	37.5	11.50	891	30.7	11.37	−0.13
4	503	255	37.0	11.79	925	31.9	11.86	+0.07
5	508	275	37.0	11.76	895	31.8	11.46	−0.30
6	528	265	38.7	12.48	944	32.2	12.75	+0.27
7	528	275	40.0	12.50	908	31.2	12.45	−0.05
8	542	290	42.7	12.28	880	28.8	12.68	+0.40
9	480	260	36.1	10.24	869	28.4	10.59	+0.25
10	504	270	41.2	11.64	838	28.2	11.43	−0.21
11	500	245	36.5	11.65	940	31.9	12.02	+0.37
12	545	270	41.5	12.96	933	31.2	13.43	+0.47
13	522	260	43.0	12.88	869	30.0	12.62	−0.26
14	520	255	38.5	12.63	944	32.8	12.67	+0.04
15	508	250	38.4	12.54	920	32.6	12.22	−0.32
16	524	275	39.7	12.25	904	30.8	12.25	+0.00
17	500	265	37.7	11.13	889	29.6	11.40	+0.27
Mean	1.0515	1.0265	39.0	12.05	1.0906	30.9	12.12	+0.08
S.D. from mean								±0.26
Maximum + deviation from HbCO								+0.47
Maximum minus deviation from HbCO								−0.32

alone, assuming that the plasma has the mean normal gravity, G_P = 1.0269. Equation 6 then simplifies to

$$\text{Hb}_B = 480(G_B - 1.0269) \tag{7}$$

The maximal error in such bloods, introduced by a deviation of ±0.002 from the assumed G_P of 1.0269, is about ±0.5 gm. of Hb per 100 ml. of blood, or 3 per cent of normal Hb_B. The data in Table IV indicate that the observed error is of this order in normal bloods, and in simple anemia, due to iron deficit or blood loss, of the type of the blood donors in Table II. When varied pathological conditions cause wide fluctuations in plasma protein concentration and G_P, however (last column, Table IV), greater errors can arise in Hb_B estimations made from G_B alone. Each gm. per 100 ml. of plasma by which the plasma proteins deviate above or below 7.4 gm. per 100 ml. causes a plus error in the same direction of about 0.7 gm. per 100 ml. of blood in the hemoglobin estimated by Equation 7. The latter can be used to follow hemoglobin changes in subjects that have been found to have plasma gravities within the range 1.025 to 1.029, and such use permits following Hb changes with finger blood. The extent of error that could arise, however, from undetected abnormality in plasma gravity is shown by a case with 12.7 gm. of plasma protein per 100 ml. reported by Atchley, Bacon, Curran, and David (3), in which the Hb_B calculated by Equation 7 was 4.7 gm. per 100 ml. of blood too high. Forty-nine other cases reported by Atchley *et al.* (3) from a general medical ward showed a distribution of error (estimated as the deviation of Hb_B by Equation 7 from Hb_B by Equation 6) similar to that of the last column of Table IV, 94 per cent of the cases showing an error of less than 1.5 gm. of Hb per 100 ml.

Calculation of Hematocrit Values from G_B and G_P

In Ashworth's (1, 4) papers, and in preliminary reports by the authors (5), Equation 3 with 1.097 as constant value of G_C was given as a means for approximate calculation of V_C from G_B and G_P, although it was recognized that the gravity-calculated V_C showed, from hematocrit-determined V_C, percentage deviations that were much greater, especially in pathological blood, than the deviations between gravity-calculated and chemically determined Hb_B values. The reason for such V_C deviation is obvious from comparison of Equations 3 and 6. If the cell gravity deviates from the assumed 1.0964, its effect on gravity-calculated V_C is proportional to the change in the factor $1/(G_C - G_P)$, and is not canceled, as in Equation 6, by parallel change of a factor in the numerator. For example, in the blood of lowest G_C in Table II, Subject 10, the gravity-calculated Hb_B agrees with that gasometrically determined within the limits of error, while the V_C value calculated by Equation 3 is 0.339, 18 per cent below 0.412 obtained by hematocrit. In normal blood V_C can be calculated by Equation 3 within ±0.02 (2 ml. of cells per 100 ml. of blood) of the hematocrit-determined value, a ±5 per cent maximal error, but the error increases so greatly, even in the moderately abnormal blood of subjects of the type in Table II, that calculation of V_C from gravities cannot be recommended, except in blood with normal cells.

EXPERIMENTAL

Blood was drawn with syringes from the arm vein and transferred to tubes containing 0.2 mg. of heparin per ml. of blood.

The *volume of packed cells per 100 ml.* (100 V_C) was determined by the conventional technique for clinical hematocrit values by use of Wintrobe (6) tubes. The blood was centrifuged at 3000 R.P.M. for 60 minutes, the center of each centrifuge tube being 18 cm. from the axis. (When a centrifuge was tried in which the centers of the tubes were only 9 cm. from the axis, 3000 R.P.M. for an hour gave with normal blood 51 ml. of cells per 100 ml. of blood, while the centrifuge with an 18 cm. axis gave 47.) The cell column obtained by the conventional technique contains, according to Gregersen and Schiro (7), about 7 per cent of its volume of inseparable plasma. The true cell volume would therefore be somewhat lower than the measured volume, and the true normal mean cell gravity somewhat higher than the 1.0964 obtained in Table I. The cell gravities, G_C, calculated in Tables I, II, and III, are the gravities of the packed red cells plus adherent plasma. If, applying Equation 5 to the mean normal values of Table I, one decreases V_C by 7 per cent, the value obtained for average normal G_C becomes 1.1013 instead of 1.0964, and the Hb concentration of the cells would be 36.3 instead of 33.9 gm. per 100 ml.

Blood hemoglobin concentrations were determined gasometrically. The hemoglobin values of Table I were obtained by a modification of Van Slyke and Neill's (8) oxygen capacity method, with details to obtain maximal accuracy. Of each blood, soon after drawing, a portion of about 10 ml. was placed in a separatory funnel of 250 ml. capacity, and aerated by rotation as described by Stadie (9). After about 20 minutes 1 ml. was transferred to the Van Slyke-Neill manometric apparatus and the O_2 + CO content was determined. Both gases, mixed with the CO_2 and N_2, were set free from the blood by ferricyanide, the CO_2 was absorbed, and the residual O_2 + CO + N_2 was measured. From the gas thus measured the sum of O_2 + N_2 physically dissolved in blood saturated with air at the observed temperature and barometric pressure was estimated from the N_2 and O_2 solubilities in blood ((10, 11); see also Fig. 48 (12)) in order to obtain the O_2 + CO bound by the hemoglobin. This procedure was used instead of determination of the O_2 alone by absorption from the extracted gases with $Na_2S_2O_4$ solution, because the latter procedure fails to include the small amounts of CO, in the neighborhood of 0.3 volume per cent, bound as HbCO that appear to be present in normal blood regularly, at least in New York. This normal blood CO is increased by smoking, which may raise it to as high as 1 volume per cent. Only a small part of such CO is displaced by O_2 in the process of aerating. The mean oxygen capacity yielded by our results, 21.6 volumes per cent, is higher than the 20.7 previously estimated from oxygen capacity determinations ((12) p. 662) from normal men.

The determinations were done in duplicate or triplicate. The aeration in the Stadie rotator was continued during the entire period of the analyses, usually 40 to 60 minutes, so that any changes that might occur, such as formation of methemoglobin or transformation of methemoglobin present *in vivo* to active hemoglobin *in vitro*, might be detected. The accepted results were those in which consecutive analyses gave oxygen capacities within 0.05 volume per cent of each other. It has been shown that during the prolonged aeration any methemoglobin present is changed to active hemoglobin to such an extent that the oxygen capacity is 99 to 100 per cent of the total hemoglobin (13) in normal blood.

The hemoglobin values in Tables II and III were obtained by the "total hemoglobin" method of Van Slyke *et al.* (13); $Na_2S_2O_4$ is added to reduce any methemoglobin present and the carbon monoxide-binding capacity of the Hb is determined as a measure of the hemoglobin. The total hemoglobin is designated as HbCO in Tables II and III.

Specific gravities of whole blood and plasma were determined by the copper sulfate method previously described (2).

The values for cell gravities and cell hemoglobin concentration are calculated in Table I to obtain from normal blood the constants for Equation 6. In Tables II and III they are included to indicate the nature of cell abnormalities in the bloods examined. It will be noted that the group in Table II, although composed of women apparently healthy except for mild anemia, showed more marked tendency to low blood cell gravity and cell hemoglobin than did the patients of Table III. In Table IV are summarized the results which have already been discussed in connection with Equation 7.

Accuracy of Calculation of Blood Hemoglobin Concentration from Gravities of Whole Blood and Plasma

The results in Tables II and III indicate that, in patients with various types of anemia, the maximal deviation of Hb, estimated from gravities measured by the copper sulfate method, from Hb gasometrically determined, is about ±0.6 gm. per 100 ml. of blood; this figure is indicated both by the maximal observed deviations and by estimation as twice the standard deviation. If 0.1 gm. per 100 ml. is allowed as the error of the gasometric determination, the maximal error of the gravity-determined Hb in pathological bloods of the types studied may be estimated as about ±0.5 gm. per 100 ml., or 3 per cent of the normal hemoglobin concentration.

TABLE III

Blood from Miscellaneous Hospital Patients on Medical Service

Patient No.	Directly measured				Calculated			
					Cells		Whole blood	
							Hb from G_B and G_P	
	G_B	G_P	Hematocrit, centrifuged cells per 100 ml. blood	HbCO, blood Hb by CO capacity	G_C by Equation 5	Cell Hb $= \frac{100\ \text{HbCO}}{\text{Hematocrit}}$	By Equation 6	Deviation from HbCO
	D_{25}^{25}	D_{25}^{25}	*ml.*	*gm. per 100 ml.*	D_{25}^{25}	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*
1*	1.0448	1.0285	24.0	8.25	1.0964	34.4	8.02	−0.23
2	440	273	24.2	8.43	963	34.8	8.19	−0.24
3*	468	268	28.9	9.50	960	32.9	9.74	+0.24
4	495	290	31.0	10.32	951	34.6	10.31	−0.01
5	448	249	28.0	9.59	960	34.2	9.43	−0.16
6†	289	225	9.2	3.28	920	35.7	2.94	−0.34
7‡	589	313	42.8	14.17	958	33.1	14.37	+0.20
8	389	243	20.2	6.93	965	34.3	6.86	−0.07
9	485	249	33.2	11.03	960	33.2	11.18	+0.15
10	465	250	30.4	9.86	957	32.4	10.21	+0.35
11	520	268	36.3	12.81	962	35.3	12.28	−0.53
12	501	255	35.0	11.85	958	33.8	11.76	−0.09
13	485	290	29.2	9.90	958	33.9	9.82	−0.08
14	466	228	32.8	11.48	954	35.0	10.96	−0.52
15§	543	295	37.5	12.45	956	33.2	12.57	+0.12
16‡	640	336	48.6	16.31	961	33.6	16.41	+0.11
17	623	292	49.6	16.50	959	33.3	16.70	+0.20
18	545	259	40.9	13.85	958	33.9	13.76	−0.11
19	565	291	41.0	13.56	959	33.1	13.81	+0.25
20	585	290	44.2	14.96	957	33.8	14.82	−0.14
21	425	253	24.5	7.94	955	32.4	8.20	+0.26
22	531	290	36.0	12.25	959	34.0	12.12	−0.13
23	482	267	31.2	10.66	960	34.2	10.52	−0.14
24	470	244	32.0	11.05	950	34.5	10.64	−0.41
25	532	290	36.4	12.34	955	33.9	12.17	−0.17
26	515	279	34.9	11.70	955	33.5	11.68	−0.02
27‖	555	270	41.6	13.75	955	33.1	13.92	+0.17
28‖	531	247	40.0	13.69	957	34.2	13.43	−0.26
29‖	511	260	36.1	12.13	955	33.6	12.09	−0.04
30	465	273	28.4	9.66	949	34.0	9.42	−0.24

TABLE III—*Concluded*

Patient No.	Directly measured				Calculated			
					Cells		Whole blood	
							Hb from G_B and G_P	
	G_B	G_P	Hematocrit, centrifuged cells per 100 ml. blood	HbCO, blood Hb by CO capacity	G_C by Equation 5	Cell Hb $= \frac{100\ \text{HbCO}}{\text{Hematocrit}}$	By Equation 6	Deviation from HbCO
	D^{25}_{25}	D^{25}_{25}	*ml.*	*gm. per 100 ml.*	D^{25}_{25}	*gm. per 100 ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*
31¶	570	262	44.2	14.77	952	33.3	14.74	−0.03
32¶	585	268	45.9	15.29	959	33.3	15.45	+0.16
33¶	572	260	44.9	14.97	955	33.2	15.02	+0.05
34¶	545	266	40.1	13.41	962	33.4	13.55	+0.14
35¶	554	260	42.1	13.64	959	32.2	14.15	+0.51
36¶	571	260	44.5	14.87	958	33.4	14.95	+0.08
37	473	297	26.6	9.00	959	33.8	8.95	−0.05
38	428	280	22.0	7.42	952	33.7	7.34	−0.08
39	421	242	25.1	7.93	954	31.6	8.41	+0.48
40	470	271	29.0	9.97	957	34.4	9.73	−0.24
41	526	290	35.2	11.64	960	33.1	11.87	+0.23
42§	485	274	31.1	10.41	952	33.3	10.36	−0.05
43§	389	273	17.0	6.41	956	37.7	5.69	−0.72
44	562	269	42.2	13.96	963	33.1	14.29	+0.33
45	569	266	43.7	14.79	960	33.9	14.71	−0.08
46	509	252	36.3	12.37	960	33.9	12.24	−0.13
47	473	258	30.9	9.81	953	31.7	10.32	+0.51
Mean deviation from HbCO								−0.02
S.D. from mean deviation								±0.26
Maximum +deviation from HbCO								+0.51
" minus deviation from HbCO								−0.72

* Pernicious anemia.

† Advanced nephritis; deficit of both hemoglobin and plasma protein.

‡ Myeloma; high plasma proteins.

§ Hodgkin's disease.

‖ Cirrhosis of the liver.

¶ Hepatitis.

The error is about the same in blood with low as in blood with high hemoglobin concentration; it is an error constant in gm. per 100 ml. rather than in percentage of the hemoglobin present.

An important part of the error in hemoglobin calculated by Equation 6 from gravities in Tables I, II, and III is attributable to the limit of error of the gravity measurements by the copper sulfate method. The greatest error in Hb occurs when errors in G_B and G_P occur in opposite directions. If a plus-minus error in G_B exceeding the standard error[1] occurs once in 3 times, a plus error equal to the standard error occurs once in 6 times. Similarly a minus error in G_P equal to the standard error occurs once in 6 times. Simultaneous occurrence of the plus error in G_B and the minus error in G_P would occur once in 36 times, and the reverse combination also occurs once in 36, so that errors in Hb from gravity errors exceeding their standard deviations, and in opposite directions, would occur once in 18 times. If the "maximal" error is taken as one that would be exceeded once in 20 times, the maximal error of Hb calculated by Equation 6 would therefore approximate (slightly exceed) the summated effect of opposite errors in G_P and G_B equal to their standard deviations, which have been found to be ±0.0003 and ±0.0004 gravity unit respectively (2). The error in Hb_B caused by errors of +0.0004 in G_B and −0.0003 in G_P is approximately 0.3 gm. of Hb per 100 ml. of blood, which is about the maximal error found in gravity analyses of normal blood (Table I). It appears that nearly all of the error of Hb_B calculated from G_B and G_P in normal subjects (Table I) is attributable to the error of the copper sulfate gravity method, and about half the error in the abnormal bloods (Tables II and III). A gravity method free

TABLE IV

Error of Hb Calculated from G_B Alone As Hb = 480(G_B − 1.0269)

Source of data	Table I	Table II	Table III
Type of subjects	Normal men	Rejected women blood donors	Hospital patients
Total No. of subjects	20	17	47
	per cent	*per cent*	*per cent*
Subjects with error under 0.5 gm.	90 (18)*	94 (16)	62 (29)
" " " " 1.0 "	100 (20)	100 (17)	81 (39)
" " " " 1.5 "			92 (44)
" " " " 2.0 "			100 (47)
	gm. Hb per 100 ml.	*gm. Hb per 100 ml.*	*gm. Hb per 100 ml.*
Mean error	+0.03	−0.01	+0.05
S.D. of error from mean	±0.27	±0.28	±0.76

* The figures in parentheses denote the number of subjects.

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[1] Standard error = standard deviation of gravity by $CuSO_4$ method from gravity by pycnometer. By the probability equation, 0.317, or 1/3.15, of the gravities by $CuSO_4$ may be expected to deviate from pycnometric gravities by more than the standard error.

of error could be expected to reduce the "maximal" Hb error in normal blood to nearly zero, and in pathological blood to 0.2 or 0.3 gm. per 100 ml.

In Fig. 1, for convenience in rapid calculation, is a nomogram prepared by standard methods (14) for calculation of plasma protein and blood hemoglobin concentrations from gravities. The plasma values are calculated by Equation 3 of the preceding paper (15), the hematocrit and hemoglobin values by Equations 3 and 6 respectively of this paper.

SUMMARY

In twenty normal and 64 abnormal, mostly anemic, heparinized human bloods, specific gravities were determined by the copper sulfate method. From specific gravities of the whole blood and plasma, blood hemoglobin concentrations were calculated by the formula of Ashworth and Tigertt, and were compared with hemoglobin concentrations determined by precise gasometric methods.

The standard deviation of the gravity-determined from gasometric hemoglobin was ±0.15 gm. per 100 ml. of blood in the normal bloods, and ±0.26 gm. in the abnormal.

A portion of the deviation equal to nearly all that observed in the normal bloods is estimated to be attributable to the limit of accuracy of the copper sulfate gravity method.

A nomogram is presented for rapid calculation of plasma protein and blood hemoglobin concentrations from specific gravities.

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THE ACTIVITY OF CERTAIN SUBSTITUTED INDOLEACETIC ACIDS AS PLANT HORMONES IN THE PEA TEST

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Although Went and his associates imply that the introduction of a methoxy group to an indolealkanoic acid effects its inactivation as a growth factor in the pea test for plant hormones (1, 2), this phenomenon has been observed only in the case of the 5-, 6-, and 7-methoxy derivatives of indolepropionic acid (2). Therefore, it seemed worth while to examine, at least qualitatively, the behavior in this test of the homologous 5-, 6-, and 7-methoxyindoleacetic acids, the synthesis of which has been described elsewhere (3). It has been found that treatment of etiolated pea stems, prepared for experiments essentially as described by van Overbeek and Went (4), with solutions of these substituted indoleacetic acids causes unmistakable inward curvatures characteristic of stem growth. Such curvatures are thought to ensue because under the experimental conditions the inner, cut surface has become insensitive to auxin-like substances; thus when the cut stems are immersed in solutions of such compounds only the outer, intact surface responds and the resulting growth differential is manifest as an inward curvature (5). The curvatures observed were of approximately the same degree as those obtained with indoleacetic acid. This is apparent from Fig. 1 where the stems treated with 5- and 6-methoxy derivatives are compared with those treated with indoleacetic acid itself and with tap water. One concludes that the inactivation produced by the introduction of a methoxy group may be peculiar to indolepropionic acid.

It is noteworthy also that 1- and 3-naphthazoleacetic acids (Fig. 2) cause growth curvatures in etiolated pea stems. However, Δ′-cyclopenteneacetic acid which satisfies all of Went's structural requirements for activity in this test (2) is apparently inert.

EXPERIMENTAL

Etiolated Pea Seedlings—Alaska peas (kindly provided by the Bristol Seed Company), after being soaked for 6 hours in tap water, were planted in ice box dishes each containing 5 pounds of washed sea sand moistened with 400 ml. of water. The dishes, each containing twenty-eight seeds, were placed under boxes in a closet until the seedlings had attained a length of 12 to 15 cm. This required 6 days in midsummer.

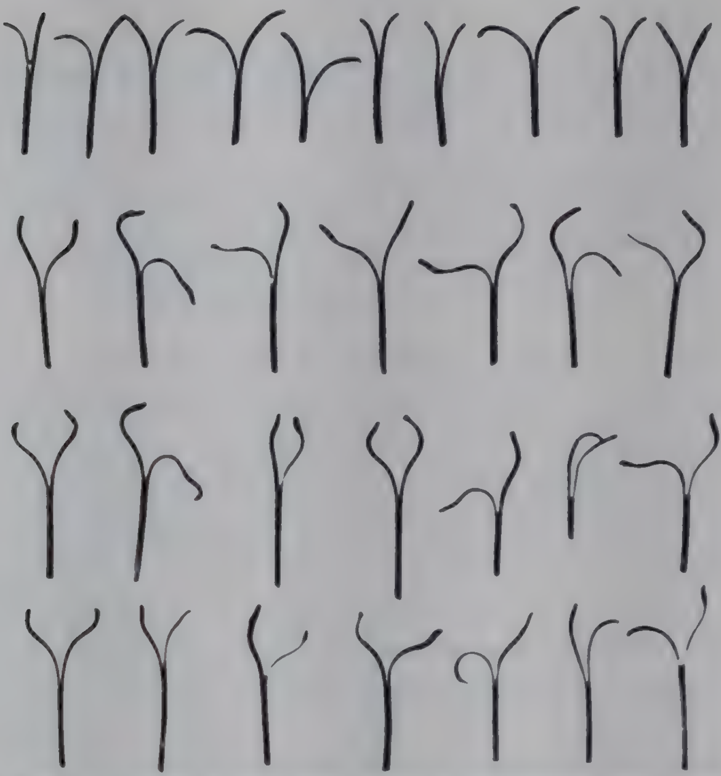

FIG. 1. Top row, etiolated pea stems after longitudinal splitting and several hours immersion in water (no apparent inward curvature). Second row, etiolated pea stems after several hours in 10^{-5} M 3-indoleacetic acid, the control. Third row, stems after similar interval in 10^{-5} M 5-methoxy-3-indoleacetic acid. Bottom row, stems after immersion in 10^{-5} M 6-methoxy-3-indoleacetic acid.

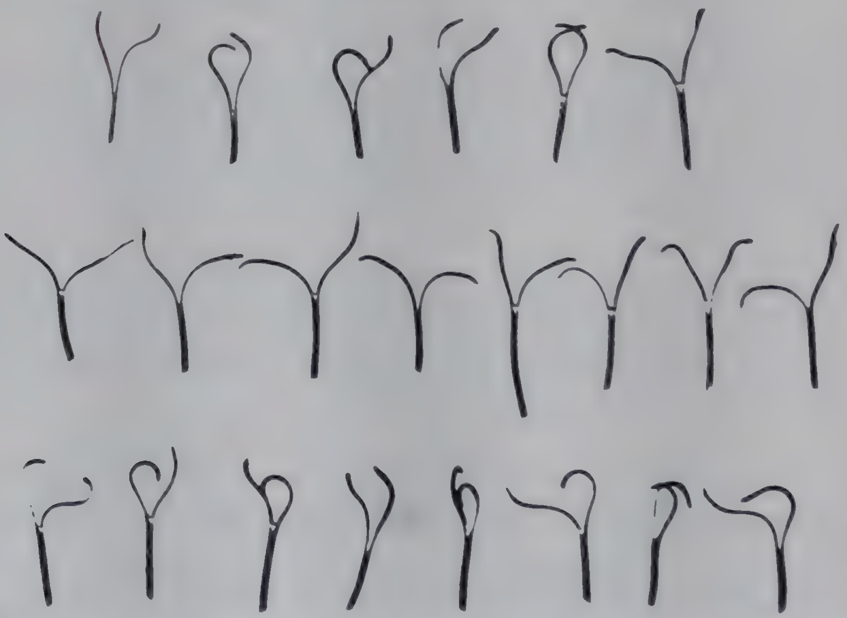

FIG. 2. Top row, etiolated pea stems after several hours immersion in 10^{-5} M 3-indoleacetic acid, the control. Middle row, stems after immersion in 10^{-5} M 3-naphthazoleacetic acid. Bottom row, stems after immersion in 10^{-5} M 1-naphthazoleacetic acid.

Preparation and Treatment of Stems—The seedlings were prepared for testing approximately as described by van Overbeek and Went (4) (*cf.* Figs. 1 and 2). After being washed for 2 hours in tap water in the dark, the stems were immersed 8 to 16 hours in 25 ml. volumes of test solution contained in Petri dishes, five to eight stems to a dish.

In each experiment, two compounds were examined for activity in comparison with indoleacetic acid as a control, and for each of the three substances dilutions of 10^{-5} and 10^{-6} M were used.

Preparation of Test Solutions—Stock solutions were prepared before each experiment by dissolving 1.0×10^{-4} mole of the control or test substances in 0.84 ml. of 0.120 N sodium hydroxide and 0.5 ml. of 95 per cent ethanol and diluting to 100 ml. The stock solution (1.0 ml.) diluted to 100 ml. provided the 10^{-5} M dilution and the appropriate treatment of this furnished the 10^{-6} M dilution.

TABLE I

Response of Pea Seedling Stems to Auxin-Like Substances

Compound	Concentration of solution	No. of stems	No. of stems responding positively
	M		
3-Indoleacetic acid (control)	10^{-5}	49	49
" " "	10^{-6}	36	36
5-Methoxy-3-indoleacetic acid	10^{-5}	19	17
" "	10^{-6}	11	11
6-Methoxy-3-indoleacetic "	10^{-5}	7	7
" "	10^{-6}	2	2
7-Methoxy-3-indoleacetic "	10^{-5}	20	12
" "	10^{-6}	8	0
1-Naphthazoleacetic acid	10^{-5}	16	16
" "	10^{-6}	14	14
3-Naphthazoleacetic "	10^{-5}	14	6
" "	10^{-6}	9	0
Cyclopenteneacetic acid	10^{-5}	14	0
" "	10^{-6}	14	0

Recording of Results—The stems, after overnight immersion in the solutions of auxin-like compounds, were freed of excess water, examined for response, and, when desirable, printed on high contrast, glossy surface Kodabromide paper (see Table I and Figs. 1 and 2).

SUMMARY

It has been found that, unlike the analogous derivatives of indolepropionic acid, 5-, 6-, and 7-methoxyindoleacetic acids act as plant hormones

in the pea test. Also, 1- and 3-naphthazoleacetic acids are active in this test, but Δ′-cyclopenteneacetic acid is not.

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STUDIES ON THE EXCRETION OF ADRENOCORTICAL COMPOUNDS

I. ISOLATION OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE AND OTHER COMPOUNDS FROM THE URINE OF NORMAL MALES*

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Although it can be said with reasonable certainty that the adrenocortical compounds elaborated by man will prove to be qualitatively similar to those already isolated from the adrenal glands of animals, it would be desirable to obtain evidence of a more direct sort. Necessarily, the approach to this problem has been indirect and has largely centered about the isolation and identification of a number of metabolic (degradation) products excreted in the urine. These compounds include both a wide variety of C-19 17-ketosteroids and a small number of C-21 compounds, which have been generally and in some instances closely associated with adrenocortical function (see Engstrom (2); Dorfman (3); Heard (4)).

Urine normally contains, in addition, what appears to be a very different group of substances, all included in what is usually termed the "cortin" fraction. Although the identity of the substances comprising this fraction remains unknown, both its chemical behavior and biological activity (see Heard (4)) suggest that it consists of a mixture of compounds of the adrenocortical type which have "escaped" metabolic degradation. This view was in part substantiated when Venning, Hoffman, and Browne (5) isolated a crystalline ketone, m.p. 234–236°, from postoperative urine which reduced alkaline silver and showed biological activity (promotion of glycogen storage in the liver). More recently, evidence of another sort was added when Mason and Sprague (6) described the recovery of appreciable amounts of 17-hydroxycorticosterone from the urine of a patient who had Cushing's syndrome and associated severe diabetes.

It is the purpose of this communication to describe a procedure for the isolation of compounds of the adrenocortical type from human urinary sources. The method includes extraction with chloroform, followed by recovery of the ketonic fraction, its partitioning between benzene and water, and chromatography of the acetylated residues. By this procedure it has been possible to isolate five crystalline compounds of the adrenocortical type and a sixth in a non-crystalline state.

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*A preliminary announcement has appeared (1).

Procedures and Methods

Collection and Extraction—Urine was collected daily over chloroform in amounts of from 20 to 30 liters from a group of male medical students.

Prior to any adjustment of pH the fresh, pooled urine was extracted by shaking three to four times with 0.1 volume of chloroform[1] to give a free (*i.e.* unconjugated) fraction. The pale yellow extracts were combined and concentrated to a small volume at low temperature *in vacuo*, and the concentrates so obtained stored as such in the cold. In some instances the urine was stored over chloroform in the cold for periods up to 12 hours and then extracted as indicated above.

After the removal of the free fraction, the pH of the urine was adjusted to 1 by the addition of concentrated hydrochloric acid, and over the course of the next 3 hours it was reextracted three to four times with chloroform. These extracts were combined and treated in the manner employed for the free fraction. The crude dark brown material so obtained was designated Fraction C-1 and was assumed to consist largely of those originally conjugated compounds relatively easily hydrolyzed by hydrochloric acid. Finally, the acidified urine was reextracted three to four times after standing at pH 1 an additional 24 hours. The dark red extract obtained in this case was assumed to consist of those substances relatively slowly liberated from the conjugated form by hydrochloric acid and was designated Fraction C-2.

At weekly intervals the accumulated chloroform concentrates were appropriately pooled, brought to a volume of about 2000 ml., and while still cold washed twice with 0.2 volume of cold 0.1 N NaOH and twice with distilled water. The extracts were then carefully reduced to dryness at low temperature *in vacuo* and stored in the cold under nitrogen. At the end of the collection period similar dried residues were combined to give a light brown, largely crystalline free fraction, a red-brown semicrystalline Fraction C-1, and a dark red, resinous Fraction C-2. By this procedure a total of 1000 liters of urine was extracted over a period of 10 weeks.

70 Per Cent Alcohol-Petroleum Ether Fractionation—As an initial step, designed both to reduce the weight of the crude fractions and in part to eliminate emulsion-stabilizing substances, the crude extracts were divided into 70 per cent alcohol-soluble and petroleum ether-soluble parts. This procedure was carried out by taking up the crude material in from 5 to 10 volumes of absolute alcohol, adding an equal volume of petroleum ether, and shaking and separating the layers after the addition of sufficient water to adjust the alcohol to 70 per cent (by volume) concentration.

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[1] Merck's U. S. P. grade, distilled after standing over anhydrous potassium carbonate.

The alcoholic phase was reextracted three times with 0.3 volume of petroleum ether and the petroleum ether phases combined and extracted twice with 0.2 volume of 70 per cent alcohol. The respective solutions were taken to dryness *in vacuo* at low temperature.

Benzene-Water Partition and Girard Separation—The procedure of benzene-water partitioning employed is based on the method described by Mason, Myers, and Kendall (7). In the case of adrenal extracts, in which the ratio of ketonic to non-ketonic elements is relatively high, these authors demonstrated that the procedure could be effectively applied to relatively crude fractions. The same procedure applied as such to crude urinary extracts, in which the ketonic-non-ketonic ratio is very low, is ineffective and becomes useful only when the non-ketonic elements (an important member being caffeine[2]) are removed. In these initial experiments, the over-all procedure consisted of dividing the 70 per cent alcohol-soluble fraction into benzene-soluble and water-soluble parts, followed by recovery of the ketonic moieties of each and their extensive partitioning between benzene and water.[3]

In each case the initial division into benzene-soluble and water-soluble components was carried out by taking up the crude 70 per cent alcohol-soluble material in approximately 100 ml. of benzene, and extracting this fifteen times with equal volumes of distilled water. Both phases were reduced to dryness *in vacuo*. The water residue contained large amounts of caffeine, but was substantially lighter in color than the benzene residue.

The ketonic parts of both residues were then separately isolated with Girard's Reagent T under mild conditions. To do this each crude fraction was taken up in 10 to 20 ml. of a 50 per cent (by volume) solution of glacial acetic acid in 90 per cent methanol. An amount of Girard's reagent equal to 5 times the weight of the crude fraction was added and the whole warmed sufficiently to achieve solution. The mixture was then allowed to stand at room temperature for 12 to 18 hours, after which there were added ice, chloroform, and, with shaking, sufficient cold 5 N NaOH to neutralize the acetic acid. The non-ketonic elements were removed by extracting the mixture with eight 0.3 volumes of chloroform. At this point the pH of the still cold mixture was adjusted to 1 by the cautious addition of concentrated hydrochloric acid and the ketonic fraction removed by repeated extraction with chloroform, both immediately and at intervals up to 24 hours. In each instance the non-ketonic fraction was twice retreated with Girard's reagent to yield additional, small ketonic fractions which were combined with the original. The neutral ketonic fraction was ultimately obtained by washing the combined chloroform solutions with dilute, cold sodium carbonate solution and water and evaporating *in vacuo*.

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[2] Although a diketone, caffeine fails to react as such under the conditions of the Girard separation here employed and regularly appears in the non-ketonic fraction.

[3] In future experiments it would appear more desirable to eliminate the initial division into benzene-soluble and water soluble parts and to partition the ketonic fraction directly.

In carrying out the benzene-water partitioning, the benzene-soluble and water-soluble ketonic fractions were separately taken up in 25 ml. of benzene and extracted with fifteen 25 ml. portions of distilled water. The benzene-soluble phases were combined and reduced to dryness to become the first benzene residue. The water phases were combined, concentrated to from 50 to 100 ml.[4] at low temperature *in vacuo*, and in turn extracted fifteen times with equal volumes of benzene. The water phase was reduced to dryness to become the first water residue. The partitioning procedure was then continued in such a fashion as to yield ultimately three benzene residues, two water residues, and a third fraction (Fraction III of Mason, Myers, and Kendall (7)) which passed readily from water to benzene and from benzene to water, here designated the benzene-water fraction. In each instance the procedure served to concentrate the greater part of the colored materials in the benzene residues and to lighten the color of the benzene-water fraction progressively.

Chromatography—Prior to chromatography the various ketonic fractions were acetylated by allowing them to stand for 18 to 24 hours in a few ml. of a pyridine-acetic anhydride mixture. The greater part of the reagents was removed with a stream of nitrogen and the remainder by washing a chloroform solution of the residue with cold 2 N HCl, dilute cold sodium carbonate solution, and water.

A 1:1 mixture of magnesium silicate[5] and Celite[6] was used as the adsorbent in a column which was in most cases roughly 5 to 10 times as long as wide. The ratio of adsorbent mixture to acetylated ketonic fraction varied from 50:1 to 100:1. Solvents were forced through the column by positive (nitrogen) pressure. In most cases the chromatography procedure consisted of applying a benzene solution of the neutral acetylated mixture to the column prepared with petroleum ether, followed by development with petroleum ether, petroleum ether containing increasing amounts of benzene, benzene, benzene-ether mixtures, ether, and ether-ethyl acetate mixtures. A detailed account of the development of one chromatogram is given in Table I.

The petroleum ether used was Baker's c.p. grade and was further purified by washing with concentrated sulfuric acid and water and was distilled after drying over sodium hydroxide flakes. The benzene was Merck's analytical reagent, thiophene-free grade, redistilled before use. Baker's c.p. absolute ether and ethyl acetate were used and, while not further dried, were redistilled before using.

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[4] Too great concentration of the water phases is avoided, since this tends to promote the formation of emulsions in the course of the subsequent extraction with benzene.

[5] Magnesium silicate No. 34, Philadelphia Quartz Company, Berkeley, California.

[6] Analytical filter aid prepared by Johns-Manville.

TABLE I

Chromatogram 1, Acetylated Free Benzene-Water Residue (144 Mg.)

Magnesium silicate 3.6 gm., Celite 3.6 gm. (column, 15 × 145 mm.). Eluates removed and examined in 20 ml. volumes.

Composition of eluent	Volume	Eluate
	ml.	
Petroleum ether	250	0
Benzene, 10–90% in petroleum ether	250 (Each)	0 to trace, non-crystalline
Benzene	250	0
Ether, 5% in benzene	500	0
" 10% " "	500	Trace, semicrystalline
" 20% " "	500	Crystalline, m.p. 226–228° (Compound I)
" 30% " "	500	Trace, non-crystalline
" 40% " "	500	" crystalline
" 50% " "	500	Crystalline, m.p. 234–238° (Compound VI)
" 60% " "	500	Crystalline, m.p. 234–238° (Compound VI)
" 70% " "	250	Trace, crystalline, m.p. 233–236° (Compound VI)
" 85% " "	250	Trace, non-crystalline
"	250	0
Ethyl acetate, 5% in ether	500	0
" " 10% " "	750	Crystalline, m.p. 263–266° (Compound II)
" " 20% " "	500	Trace, crystalline, m.p. 263–266° (Compound II)
" " 40–100% in ether	250 (Each)	Non-crystalline

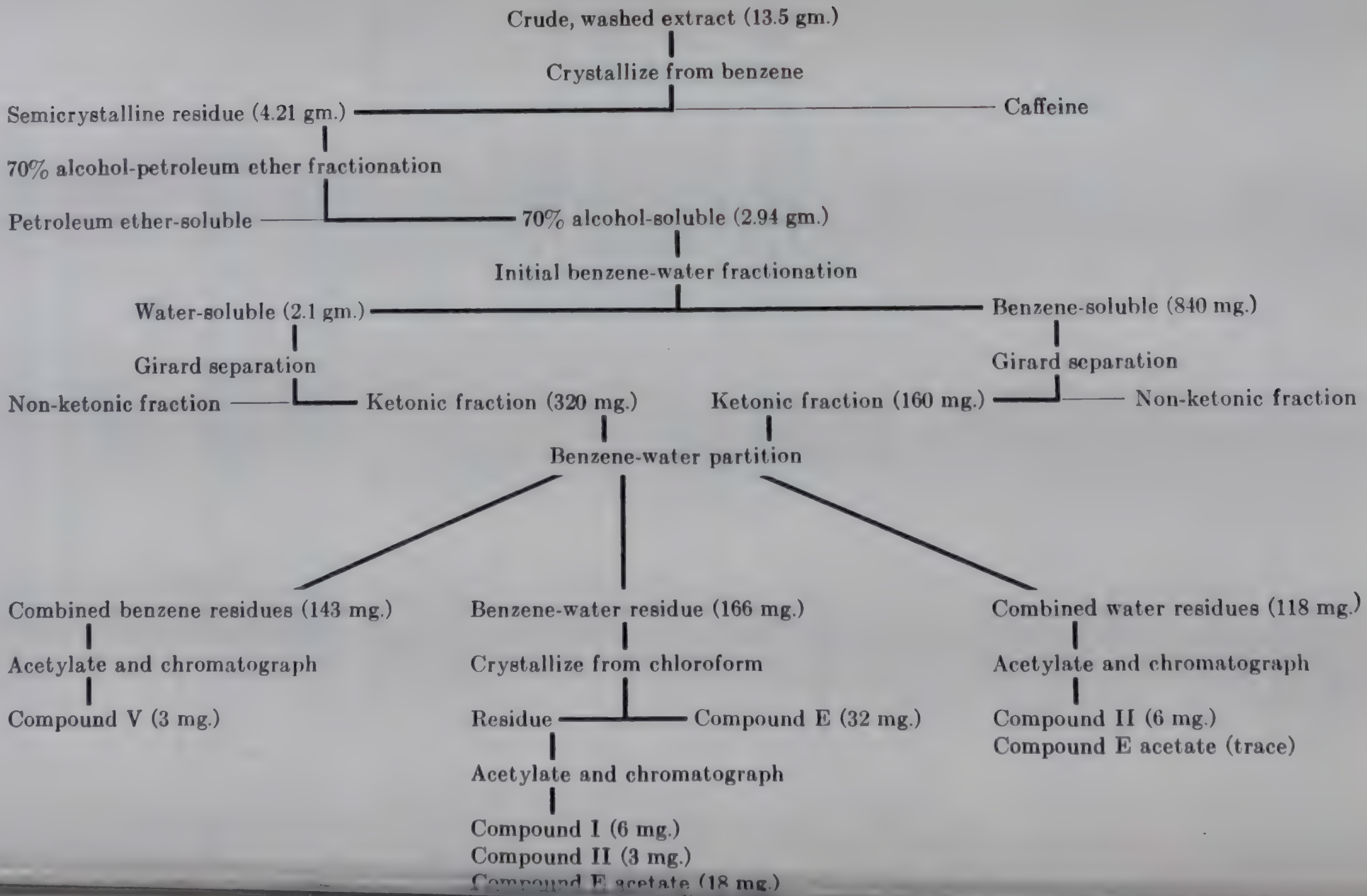
All melting points were determined on a Fisher-Johns type apparatus and are recorded as read. The absorption spectra were determined with a Beckman spectrophotometer.

Results

Fractionation of Free Fraction

The further treatment of the free fraction here detailed is given in outline form in the accompanying diagram. The same general procedure

Fractionation of Free Extract


Crude, washed extract (13.5 gm.)
Crystallize from benzene
Semicrystalline residue (4.21 gm.)
Caffeine
70% alcohol-petroleum ether fractionation
Petroleum ether-soluble
70% alcohol-soluble (2.94 gm.)
Initial benzene-water fractionation
Water-soluble (2.1 gm.)
Benzene-soluble (840 mg.)
Girard separation
Girard separation
Non-ketonic fraction
Ketonic fraction (320 mg.)
Ketonic fraction (160 mg.)
Non-ketonic fraction
Benzene-water partition
Combined benzene residues (143 mg.)
Acetylate and chromatograph
Compound V (3 mg.)
Benzene-water residue (166 mg.)
Crystallize from chloroform
Residue
Compound E (32 mg.)
Acetylate and chromatograph
Compound I (6 mg.)
Compound II (3 mg.)
Compound E acetate (18 mg.)
Combined water residues (118 mg.)
Acetylate and chromatograph
Compound II (6 mg.)
Compound E acetate (trace)

was employed for Fractions C-1 and C-2, except that in these cases no attempt was made to remove the caffeine.

Isolation of Caffeine—The tan, semicrystalline, 13.5 gm. crude free fraction was taken up in benzene, concentrated to the point of crystallization, warmed, and allowed to stand at room temperature. The material which then separated was washed with cold ethanol and recrystallized from ethanol or benzene, taking the form of long, somewhat opaque needles, m.p. 234–238°, with varying tendencies to sublime at lower temperatures. A small sample was sublimed for analysis.

TABLE II

Distribution of Crystalline Compounds in Chief Fractions

Fraction	Weight	Compounds isolated					
		I	II	III	IV*	V	VI
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Free combined benzene residues	143					3	
“ “ water “	118		6				Trace
“ benzene-water residue	166	6	3				50†
C-1, combined benzene residues	257						
“ “ water “	70						
“ benzene-water residue	202		3	6			8
C-2, combined benzene residues	343						
“ “ water “	53						
“ benzene-water residue	245			3	4		

* Non-crystalline.

† 32 mg. as the free compound; 18 mg. as the acetate.

$C_8H_{10}O_2N_4$. Calculated, N 28.86; found, N 28.40

Isolation of 17-Hydroxy-11-dehydrocorticosterone and Its Acetate (Compound VI, Table II)—Following the removal of the caffeine, the residue (4.21 gm.) was divided into petroleum ether-soluble and 70 per cent alcohol-soluble parts, and the latter (2.94 gm.) was redivided into a water-soluble portion (2.1 gm.) and a benzene-soluble portion (840 mg.). Treatment of both fractions with Girard's reagent gave water-soluble ketonic fractions totaling 320 mg. and benzene-soluble ketonic fractions totaling 160 mg. Both fractions were partitioned between benzene and water (by combining appropriate fractions) to yield three benzene residues totaling 143 mg., two water residues totaling 118 mg., and a single benzene-water residue of 166 mg.

In the course of reducing a chloroform solution of the free benzene-water residue to dryness, crystalline material separated which proved to be free 17-hydroxy-11-dehydrocorticosterone (Kendall's Compound E, Reichstein's Substance Fa; (preliminary communication (1)). In addition to the 32 mg. recovered as the free steroid, 18 mg. of the corresponding acetate have since been isolated in the course of chromatographing the acetylated residues of the fraction it was obtained from. The compound was eluted upon the application of 40 to 70 per cent ether in benzene (by volume) solutions to a magnesium silicate-Celite column of the residue (Chromatogram 1), a detailed description of which is given in Table I.

The acetate crystallized from absolute ethanol in the form of needles, melted at 238–240° (after becoming opaque at around 200°), and showed no depression in melting point on admixture with an authentic preparation of 17-hydroxy-11-dehydrocorticosterone acetate. The addition of sulfuric acid to a small amount of the crystalline compound gave a yellow solution with a faint transient green fluorescence. Methanolic solutions of the acetate reduced ammoniacal silver at room temperature (somewhat more slowly than the free compound) and formed a red precipitate upon the addition of a few drops of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl (Brady's reagent).

Isolation of Compound I (Table II)—This compound was the first eluted from the column described in Table I. It was removed upon the application of a 10 to 20 per cent (by volume) solution of ether in benzene. Upon recrystallization from methanol it took the form of plates, m.p. 226–228°. Methanolic solutions reduced alkaline silver promptly and slowly gave a yellow-orange precipitate upon the addition of a few drops of Brady's reagent. When mixed with sulfuric acid, the crystals formed a yellow nonfluorescing solution.

Isolation of Compound II (Table II)—Small amounts of this compound were obtained from both the free benzene-water residue (Chromatogram 1, Table I) and the free water residues. In each case the compound was eluted upon the application of a 10 per cent (by volume) solution of ethyl acetate in ether and, when recrystallized from absolute ethanol, took the form of short needles. The melting point of the various fractions proved to be the same, 265–268°, with marked decomposition. Admixture of the crystalline substance with concentrated sulfuric acid generally gave light brown solutions. In methanolic solution the compound formed a red-brown and then a black precipitate when tested with alkaline silver and formed a red precipitate upon the addition of a few drops of Brady's reagent. The absorption spectrum as measured in the ultraviolet region was very similar to that obtained in the case of 17-hydroxy-11-dehydrocorticosterone, with a maximum at 237–238 mμ.

Isolation of Compound V (Table II)—This substance, which has been obtained in very small amounts, is the only one as yet isolated from a benzene residue fraction. It was recovered in the course of chromatographing the acetylated free combined benzene residues and was eluted upon the application of a 70 per cent (by volume) solution of benzene in petroleum ether. It was recrystallized from absolute ethanol to give short, fine needles, m.p. 228–230°. Like Compound I, it immediately reduced alkaline silver and gave an orange-yellow solution on treatment with Brady's reagent. On admixture with sulfuric acid it formed a yellow-brown solution.

Fractionation of Fractions C-1 and C-2

Isolation of 17-Hydroxy-11-dehydrocorticosterone Acetate (*Compound VI, Table II*)—An additional 8 mg. of 17-hydroxy-11-dehydrocorticosterone acetate were isolated in the course of chromatographing the acetylated benzene-water Fraction C-1. The properties of this sample were in every regard the same as those of the corresponding acetate isolated from the free fraction.

The over-all yield of 17-hydroxy-11-dehydrocorticosterone was thus raised to 55.3 mg., corresponding to 55.3 γ per liter.

Isolation of Compound III (*Table II*)—This substance was recovered in the course of chromatographing the benzene-water Fractions C-1 and C-2 and in each case was eluted upon the application of a 20 to 30 per cent (by volume) solution of ether in benzene. The crude material was readily recrystallized from absolute ethanol to give colorless, flat needles, m.p. 224–226°. Like Compounds I and V, this substance reduced ammoniacal silver in the cold (giving, however, first a brown, then a black precipitate) and gave a yellow-orange precipitate when treated with Brady's reagent. The melting point on admixture with Compound I (m.p. 227–228°) was from 222–226° to 224–226°. It differed from Compound I, however, in crystal form (needles rather than plates) and in the sulfuric acid test, the addition of sulfuric acid giving a brown to red-brown solution in contrast to the yellow solution characteristic of Compound I. On admixture[7] with Reichstein's Substance V acetate (allopregnane-3(β),11(β),17(β)-21-tetrol-20-one diacetate; needles, m.p. 223–224°) the melting point was 210–216°. While it is recognized that Compounds I and III may represent mixtures, they are tentatively regarded as different substances. Compound V is tentatively regarded as a distinct compound and different from Compounds I and III because it appeared in a different fraction and because it was eluted from the column well before the other two substances would be expected to appear.

Isolation of Compound II (*Table II*)—An additional 3 mg. of this compound, previously isolated from the free water residues and the free benzene-water fraction, were obtained when chromatographing the benzene-water Fraction C-1. It was eluted at the same point and was in all other regards identical with the acetate previously described.

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[7] The author wishes to thank Professor T. Reichstein, Pharmazeutische Anstalt der Universität, Basel, for generously supplying a sample of his Substance V acetate.

Isolation of Compound IV (Table II)—This substance was isolated in a non-crystalline state in the course of chromatographing the acetylated benzene-water Fraction C-2. It was eluted with highly colored material upon washing the column with a 60 per cent (by volume) solution of benzene in petroleum ether. Methanolic solutions of the crude fraction immediately reduced alkaline silver and immediately formed a red precipitate upon the addition of Brady's reagent.

DISCUSSION

It is evident from these initial experiments that the recovery of compounds of the adrenocortical type from urinary sources is a feasible procedure. While lengthy, the isolation process is relatively simple and the conditions imposed sufficiently mild to preclude largely the possibility that the substances recovered are transformation products. It is to be expected that, with refinement in method, higher yields and a greater number of crystalline compounds may be obtained. The method as here outlined may be applied to the fractionation of urine obtained in pathological states, in pregnancy, and to urine from animal sources.

These data demonstrate that adult males normally excrete small amounts of 17-hydroxy-11-dehydrocorticosterone and lend further support to the thesis that the human adrenal gland elaborates this substance. Qualitatively and quantitatively, this compound could of itself largely account for the biological (glyconeogenic) activity of the cortin fraction. For example, Heard, Sobel, and Venning, using the glycogen deposition method, have reported (8) that normal adult males excrete from 45 to 77 γ of cortin substance per day (expressed in terms of Compound E). In these experiments, the over-all recovery of Compound E may be roughly estimated to be 82 γ per 24 hours, assuming an average 24 hour output of 1500 ml. of urine.

While it is difficult to evaluate isolation experiments in quantitative terms, it seems reasonable to predict that other physiologically active compounds, if found, will be present in comparatively small amounts. Since Compounds II and IV appear to possess two of the structural requisites (the α,β-unsaturated system and the α-ketol side chain) necessary for biological activity, they too may ultimately prove to be active.[8]

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[8] In addition to promoting glycogen deposition, the ability of the cortin fraction to prolong the survival period of the adrenalectomized rat (5, 9) and to increase the tolerance of such animals to cold (10) suggests that it may contain compounds of the 11-desoxy series. If such are present, it may be expected (7) that they would appear in the benzene residues. Neither Compound II nor IV appeared in such a fraction.

Compounds I, III, and V are clearly reduction products. It remains to be determined whether they are elaborated in the gland as such or whether they are derived from other compounds at an extraglandular site. Since they lack the α,β-unsaturated system, it can be predicted that they will have little if any biological activity. The presence of compounds of this type in urine would in part account for the uniformly higher results given by the chemical (colorimetric) methods as contrasted to the biological assay for cortin substances.[9]

It is of interest that these substances are excreted in the free form in some cases (Compound VI (largely) and Compounds I, II, and V) and in a conjugated form in others (Compounds III and IV). The point is of considerable practical importance because it again raises the question of how hydrolysis of the conjugated forms may be most readily and safely achieved. Although acid is employed as the hydrolytic agent in these experiments, it is recognized that, while possibly effective, the procedure may well prove to be destructive to some compounds. The observation frequently made that strong acids, particularly at elevated temperatures, reduce or destroy the activity of cortin fractions suggests that 17-hydroxy-11-dehydrocorticosterone, for example, is acid-labile as well.

While no accurate comparison can be made between these data and those obtained by measuring the chemical or biological properties of extracts prepared before and after hydrolysis at pH 1, it may be noted that the results reported here do not support the statements (8, 11, 12) that hydrolysis increases the yield of extractable active material 2 to 4 times. As may be calculated from Table II, 77 per cent of the crystalline material and 86 per cent of the Compound E isolated were obtained prior to the hydrolysis procedure. The difficulties generally encountered in the treatment of acid-treated urine do not center about the amount or nature of the contaminating substances, for, although the crude extract is dark, the Girard separation together with the benzene-water partitioning effectively removes these.

The author wishes to express his thanks to Dr. Wm. Pearlman of the Department of Biochemistry of this institution for his generous assistance and valued advice, and Dr. T. F. Gallagher and Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, for many helpful suggestions and for determining the infra-red spectra of Compounds I, II, and III.

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[9] The data of Heard, Sobel, and Venning (8) include a table illustrating these differences.

SUMMARY

1. Normal male urine has been examined for compounds of the adrenocortical type by a procedure that includes extraction with chloroform, recovery of the ketonic fraction, its partitioning between benzene and water, and chromatography of the acetylated residues.

2. Of the crystalline compounds recovered, one has been shown to be 17-hydroxy-11-dehydrocorticosterone, which was isolated largely as the free compound in an over-all yield of 55.3 γ per liter.

3. Four other crystalline compounds of the adrenocortical type and one other non-crystalline substance have been partially characterized. Three of these are regarded as reduction products and the remaining two as possibly physiologically active compounds.

4. These results have been discussed in relation to other relevant data.

Addendum—Since this paper was submitted, it has been found that the infra-red absorption of Compound I is the same as that given by tetrahydro E acetate (3(α),21-diacetoxy-17(α)-hydroxypregnanetriol-11,20-dione), thus definitely establishing the identity of this compound. The author again wishes to thank Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research for his assistance in this regard.

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SYNTHETIC ACTION OF PHOSPHATASE*

II. TRANSPHOSPHORYLATION BY ALKALINE PHOSPHATASE IN THE ABSENCE OF NUCLEOTIDES

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Axelrod (1) recently reported that a direct transfer of phosphate occurred from some aryl phosphates to certain aliphatic alcohols in the presence of citrus phosphatase and some other acid phosphatases. The phosphate did not pass through the inorganic stage. This was indicated by the fact that inorganic phosphate did not esterify the acceptor compound under the conditions in which it was phosphorylated by nitrophenyl phosphate. The direct transfer was, moreover, confirmed by experiments with P^{32} (2). This phenomenon was observed not to occur with the biological sugars or in the presence of ordinary alkaline phosphatase.

We have found, however, that a similar transfer occurs readily with alkaline phosphatase and with the common biological polyalcohols (glucose, fructose, and glycerol) in the presence of phosphocreatine, phosphopyruvate, and glucose-1-phosphate. In these cases it can be observed only with P^{32}, because, in contrast to the system studied by Axelrod, the ester phosphates formed remain in enzymatic equilibrium with the inorganic phosphate and with the free polyalcohol from which the ester is formed. There is no excess formation of ester over that allowed by the thermodynamic equilibrium. The phosphate transfer proceeds only from a higher level of phosphate energy to a lower level. It is most pronounced in the system phosphopyruvate or phosphocreatine to glycerol. These transfers attain 100 per cent; that is, all synthesis of glycerophosphate over that of the control in the absence of the phosphate donor occurs by transfer. This was already surmised by us (3) when we observed an increase of several hundred per cent in the speed of synthesis of glycerophosphate in the presence of phosphocreatine. Glucose-1-phosphate can also be used as P donor; fructose and glucose instead of glycerol can be used as acceptors. We have, finally, found such a phosphate transfer from phosphocreatine

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to glycerol in an enzymatic mixture with glycerophosphate at equilibrium, if labeled phosphocreatine is added. Even if the system is not at equilibrium, by virtue of a several fold excess of glycerophosphate so that the latter breaks down to attain the equilibrium distribution, the transfer of P from phosphocreatine to glycerophosphate can still be demonstrated.

Methods

All incubations were conducted at 38°.

Phosphate was determined by the method of Fiske and Subbarow (4) as modified by Lohmann and Jendrassik (5).

Inorganic phosphate was precipitated by magnesia mixture and removed as $MgNH_4PO_4$.

The amount of synthesized organic phosphate, or, as in the equilibrium and hydrolysis studies of glycerophosphate, the amount of glycerophosphate remaining, was determined as the difference between the total organic phosphate and that of the added phosphate donor remaining. In every experiment a control incubation was run simultaneously in order to follow the rate of hydrolysis of the added phosphate donor in the absence of the material being phosphorylated. The added phosphate donor was determined by acid hydrolysis (phosphocreatine and glucose-1-phosphate) or by alkaline hypoiodite oxidation (phosphopyruvate).

Materials—

Phosphate acceptors. Merck's anhydrous glucose, Merck's crystalline levulose, Baker's analyzed glycerol.

Phosphate donors. Phosphocreatine (barium salt) labeled with P^{32} was prepared enzymatically according to the method of Meyerhof, Schulz, and Schuster (6), and recrystallized to constant purity, 96 to 97 per cent of labile phosphorus. Glucose-1-phosphate (dipotassium salt) labeled with P^{32} was prepared enzymatically according to the method of Kiessling (7). It was best crystallized by the slow addition of alcohol to the hot aqueous solution until persistent turbidity and by permitting the mixture to come slowly to room temperature. Tiny white crystals were obtained containing 99 to 100 per cent of 10 minute-hydrolyzable phosphorus. Phosphopyruvate (barium salt) was a synthetic preparation made by Kiessling (8) about 10 years ago and purified in our laboratory (9). The purity was 99 per cent by the alkaline hypoiodite method of Lohmann and Meyerhof (10).

Na-glycerophosphate (Heyden Chemical Corporation) 60 per cent α, 40 per cent β ester.

Ca-α-glycerophosphate (Bios Laboratories, Inc.) 97 per cent α ester.

Radioactive inorganic phosphate. When used in the experiments with phosphopyruvate, the sample was hydrolyzed in 2 N HCl for 20 minutes and treated with a few drops of dilute $KMnO_4$ for 5 minutes at the reflux temperature. This procedure removed the metaphosphate and phosphite, with which the orthophosphate (P^{32}) from the Isotopes Division, Oak Ridge, is often contaminated. After the treatment only a negligible amount of P^{32} not precipitated by magnesia mixture remained.

Alkaline intestinal phosphatase was purified according to Schmidt and Thannhauser (11).

Handling of Phosphates for Counting

All phosphate species were recovered in the form of $MgNH_4PO_4$. The stable organic phosphates (glucose-6-phosphate, fructose phosphate, and glycerophosphate) were decomposed by incineration or by phosphatase hydrolysis, while the labile phosphate of the added phosphate donor was split by suitable means as mentioned above. Carrier inorganic phosphate was added prior to precipitation with magnesia mixture in order to dilute the activity when necessary. The last trace of residual activity was similarly removed by the addition of carrier inorganic phosphate and subsequent removal by magnesia mixture. The precipitates of $MgNH_4PO_4$ were washed three times with 3 per cent NH_4OH and twice with acetone and plated onto a tin plate from an acetone suspension.

After having been counted, the plated samples were washed off the plates with H_2O, dissolved in dilute acid, and analyzed for inorganic phosphate.[1] From the ratio of labeled phosphate species to added carrier phosphate and from the total activity of this mixture, the specific activity of the undiluted labeled phosphate species was readily calculated: A = micrograms of P of the labeled phosphate species; B = micrograms of P of added carrier phosphate; C = micrograms of P of the counted sample; N = the activity of C. Therefore, the specific activity of labeled phosphate species = $N(A + B)/A \times C$.

EXPERIMENTAL

All incubations were carried out at 38° and pH 8.5 in a volume varying from 0.5 to 1.1 ml. All calculations in the tables are made on the basis of 1 ml.

A typical experiment on the transphosphorylation of glycerol by means of radioactive phosphocreatine is described in detail as illustrative of the general procedure. In the other experiments the conditions and procedure were essentially the same; therefore, only the modifications of this general procedure will be described.

Glycerol, Radioactive Phosphocreatine (*Table I, Experiment I*)—The following solutions were incubated at 38° at pH 8.5 for 15 minutes: (*a*) 0.1040 gm. of glycerol, 0.12 ml. of Na_2HPO_4 (2.75 M), 0.50 ml. of H_2O, and 0.05 ml. of purified intestinal phosphatase (activity, 3280 units per ml. as defined by Schmidt and Thannhauser); (*b*) same as (*a*) but containing 535 γ of P of labile radioactive phosphocreatine and 21 γ of P of non-labile radioactive organic phosphate;[2] (*c*) same as (*b*) but with the glycerol replaced by an equal volume of H_2O.

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[1] The quantitative recovery of the $MgNH_4PO_4$ from the plate in this procedure is indicated by the fact that the residual activity of the washed plate could be accounted for by the background count.

TABLE I

A. Transphosphorylation of Glycerol by P^{32}-Phosphocreatine

Experiment No.	Enzyme units per ml.	Time of incubation	Initial molar concentration			Glycerophosphate formed	Phosphocreatine split
			Glycerol	Inorganic P	Phosphocreatine		
		min.				*μM*	*per cent*
Ia	218	15	1.52	0.438	0	0.54	
Ib	218	15	1.52	0.438	0.023	1.70	18.7
Ic	218	15	0	0.438	0.023		21.5
IIa	214	30	1.63	0.447	0	1.43	
IIb	214	30	1.63	0.447	0.025	4.13	25.3
IIc	214	30	0	0.447	0.025		30.5

B. Specific Activity of (b) Samples

Counts per minute per microgram of P.

Experiment No.	Acceleration of synthesis	Phosphate species						
		Phosphocreatine			Inorganic P		Glycerophosphate	
		0 min.	15 min.	30 min.	15 min.	30 min.	15 min.	30 min.
	per cent							
Ib	218	2412	2210		15		(1738) 1487*	
IIb	188	1090		895		9		(707) 614*

* Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

The solutions were deproteinized with 1.0 ml. of ice-cold 5 per cent trichloroacetic acid, chilled, and immediately made ammoniacal with 1.0 ml. of 12 N NH_4OH. The inorganic phosphate was precipitated by magnesia mixture in a total volume of 15.0 ml. and removed by filtering through a sintered glass funnel. The filtrates were analyzed for inorganic and organic phosphate. The determination of direct phosphate under the conditions of Fiske and Subbarow as modified by Lohmann and Jendrassik gave the amount of phosphocreatine unsplit in incubations (*b*) and (*c*). In the latter sample, moreover, the difference between the total organic and phosphocreatine phosphate represented the amount of non-labile impurity of phosphocreatine remaining as stable organic phosphate. The remaining filtrate (13.0 ml.) of incubation sample (*b*), containing the radioactive glycerophosphate, was boiled to rid it of excess NH_3, acidified with 2.0 ml. of 12 N HCl, and incubated at 38° for 30 minutes in order to hydrolyze the unsplit phosphocreatine. Carrier inorganic phosphate (60 mg.) was added and the total inorganic phosphate precipitated by magnesia mixture and removed by filtration through a sintered glass funnel. This precipitate is designated as the phosphocreatine phosphate species, while the first precipitate of the inorganic phosphate is the inorganic phosphate species. The filtrate of the above was again treated with carrier inorganic phosphate (30 mg.) and the treatment with magnesia mixture repeated. The latter precipitate was discarded. The filtrate containing the glycerophosphate and non-labile phosphate from the phosphocreatine was boiled to free it from excess NH_3 and analyzed for inorganic and organic phosphate. To the remaining volume (18 ml.) were added 10 ml. of borate buffer (pH 9.1); the mixture was adjusted to pH 9.3 and incubated overnight at 38° with 1640 Schmidt units of phosphatase. The increase in the inorganic phosphate (95 to 100 per cent of the organic phosphate initially present) corresponded to the glycerophosphate species, and was derived from both the glycerophosphate and the non-labile organic phosphate originally present in the phosphocreatine. To the remaining solution containing 17 γ of P from the glycerophosphate and 9 γ of P from the non-labile impurity of phosphocreatine was added carrier inorganic phosphate (5.0 mg.), and the total inorganic phosphate was precipitated with magnesia mixture and removed as $MgNH_4PO_4$ (glycerophosphate species). As a standard for comparison of activities a sample of the stock radioactive phosphocreatine solution used in the incubations was hydrolyzed in 1 N HCl at 38° for 30 minutes, carrier inorganic phosphate added, and the total inorganic phosphate precipitated by magnesia mixture and removed as $MgNH_4PO_4$.

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[2] The non-labile organic impurity varied, in different samples, from 3 to 4 per cent of the total organic P.

All precipitates of $MgNH_4PO_4$ prior to counting were washed three times with 3 per cent NH_4OH and twice with acetone. The results are given in Table I, Experiment I. The total activity accounted for was 93 per cent (see Table II).

Fructose, Radioactive Phosphocreatine (*Table III*)—Removal of excess NH_3 by boiling and the hydrolysis of the fructose phosphates by phosphatase at 38° were performed in an atmosphere of nitrogen gas in order to avoid discoloration due to oxidation by air.

TABLE II

Calculation of Activity Balance

P species	P	Specific activity	Total activity
	γ		
Input			
Phosphocreatine (+ impurity)	546	2412	**1,315,000**
Recovered			
Inorganic	10,270	14.8	152,000
Labile phosphocreatine unsplit	435	2210	962,000
Glycerophosphate fraction (+ phosphocreatine impurity)	61	1738	106,000
Sum			**1,220,000**
Recovery, %		93	

TABLE III

A. Transphosphorylation of Fructose by P^{32}-Phosphocreatine; Incubations for 15 Minutes

Experiment No.	Enzyme units per ml.	Initial molar concentration			Fructose phosphate formed	Phosphocreatine split
		Fructose	Inorganic P	Phosphocreatine		
					μM	*per cent*
Ia	372	2.06	0.443	0	0.55	
Ib	372	2.06	0.443	0.028	1.87	12.4
Ic	372	0	0.443	0.028		30.0
IIa	372	2.06	0.443	0	0.62	
IIb	372	2.06	0.443	0.028	1.72	13.3
IIc	372	0	0.443	0.028		31.5

B. Specific Activities of (b) Samples

Counts per minute per microgram of P.

Experiment No.	Acceleration of synthesis	Phosphate species			
		Phosphocreatine		Inorganic P	Fructose phosphate
		0 min.	15 min.	15 min.	15 min.
	per cent				
Ib	227	1828	1750	13	(1190) 556*
IIb.	175	2380	2145	15	(1108) 607*

* Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

Glycerol, Radioactive Glucose-1-phosphate (Table IV)—The glucose-1-phosphate unsplit at the end of the incubation was decomposed by hydrolysis in 1 N HCl at 100° for 15 minutes. The inorganic phosphate liberated was precipitated by magnesia mixture and worked up as described.

Glycerol or Glucose, Phosphopyruvate, Radioactive Inorganic Phosphate (Table V)—In this series of experiments the transphosphorylation is demonstrated not by an excess of activity in the synthesized ester but by a lower activity compared with that of the inorganic phosphate species. The highly active inorganic phosphate fraction after removal from the incubation mixture by magnesia mixture was dissolved in dilute HCl, analyzed for phosphate, treated with a known amount of carrier inorganic phosphate, and reprecipitated as $MgNH_4PO_4$. The phosphopyruvate fraction, on the other hand, was completely free of any activity, and was, therefore, discarded after removal. The phosphopyruvate was decomposed by alkaline hypoiodite; in the presence of glucose, however, it was necessary to add an excess of hypoiodite to oxidize completely both the glucose and the phosphopyruvate.

TABLE IV

A. Transphosphorylation of Glycerol by P^{32}-Glucose-1-phosphate; Incubations for 15 Minutes

Experiment No.	Enzyme units per ml.	Initial molar concentration			Glycerophosphate formed	Glucose-1-phosphate split
		Glycerol	Inorganic P	Glucose-1-phosphate		
					μM	*per cent*
Ia	138	1.97	0.524	0	0.57	
Ib	138	1.97	0.524	0.014	1.15	42.5
Ic	138	0	0.524	0.014		53.2
IIa	93	1.92	0.509	0	0.62	
IIb	93	1.92	0.509	0.027	1.52	8.9
IIc	93	0	0.509	0.027		20.5

B. Specific Activities of (b) Samples

Counts per minute per microgram of P.

Experiment No.	Acceleration of synthesis	Phosphate species			
		Glucose-1-phosphate		Inorganic P	Glycerophosphate formed
		0 min.	15 min.	15 min.	15 min.
	per cent				
Ib	100	1935	1890	18	543
IIb	145	2400	2070	15	673

Glycerophosphate, Radioactive Phosphocreatine (Tables VI and VII)—A several fold excess of glycerophosphate over the equilibrium value was used in the hydrolysis experiment (Table VII) in order to produce a substantial hydrolysis and, at the same time, permit an accurate analysis of the glycerophosphate remaining at the end of the experiment. In addition to the control which allowed us to follow the rate of splitting of phosphocreatine in the absence of glycerol and glycerophosphate, another control containing both glycerophosphate and phosphocreatine but not glycerol was run simultaneously. The latter was included for the purpose

TABLE V

A. Transphosphorylation of Glycerol and Glucose by Phosphopyruvate with P^{32}-Inorganic Phosphate

Experiment No.	Enzyme units per ml.	Time of incubation	P acceptor	Initial molar concentration			Acceptor phosphate formed	Phosphopyruvate split
				P acceptor	Inorganic P	Phosphopyruvate		
		min.					*µM*	*per cent*
Ia	213	15	Glycerol	1.47	0.432	0	0.58	
Ib	213	15	"	1.47	0.432	0.021	4.52	65
Ic	213	15		0	0.432	0.021		77
IIa	213	15	Glycerol	1.47	0.432	0	0.63	
IIb	213	15	"	1.47	0.432	0.020	4.23	67
IIc	213	15		0	0.432	0.020		77
IId	213	150	Glycerol	1.47	0.432	0	3.27	
IIe	213	150	"	1.47	0.432	0.020	10.1	100
IIIa	365	15	Glucose	2.06	0.423	0	0.74	
IIIb	365	15	"	2.06	0.423	0.024	5.38	67
IIIc	365	15		0	0.423	0.024		82
IIId	365	150	Glucose	2.06	0.423	0	4.88	
IIIe	365	150	"	2.06	0.423	0.024	8.95	100

B. Specific Activities of (b) and (e) Samples

Counts per minute per microgram of P.

Experiment No.	Acceleration of synthesis	Phosphate species						
		Inorganic P			Glycerophosphate		Glucose-6-phosphate	
		0 min.	15 min.	150 min.	15 min.	150 min.	15 min.	150 min.
	per cent							
Ib	680	1174	1123		242			
IIb	570	587	548		120			
IIe		587		437		310		
IIIb	630	527	490				84	
IIIe		527		400				334

of determining whether any direct isotopic exchange had taken place between the radioactive phosphocreatine and the glycerophosphate in the absence of free glycerol. In both experiments (equilibrium and hydrolysis) because of the relatively large amounts of glycerophosphate present at the end of the incubation, it was necessary to wash the first precipitate of $MgNH_4PO_4$ with dilute NH_4OH to avoid a considerable loss of glycerophosphate by occlusion. Similarly, it was found more practicable to decompose the glycerophosphate fraction by incineration rather than by phosphatase hydrolysis.

TABLE VI

A. Phosphate Transfer at Enzymatic Equilibrium of Glycerophosphate in Presence of P^{32}-Phosphocreatine

Experiment No.	Enzyme units per ml.	Time of incubation	Initial molar concentration				Glycerophosphate	Phosphocreatine split
			Glycerol	Inorganic P	Phosphocreatine	Glycerophosphate*		
		min.					*M*	*per cent*
Ia	213	15	1.86	0.346	0	0.025	0.024	
Ib	213	15	1.86	0.346	0.022	0.025	0.024	8.0
Ic	213	15	0	0.346	0.022	0		10.5
IIa	213	150	1.86	0.346	0	0.025	0.024	
IIb	213	150	1.86	0.346	0.022	0.025	0.023	50.0
IIc	213	150	0	0.346	0.022	0		68.0

B. Specific Activities of (b) Samples

Experiment No.	Phosphate species						
	Phosphocreatine			Inorganic P		Glycerophosphate	
	0 min.	15 min.	150 min.	15 min.	150 min.	15 min.	150 min.
Ib	1240	1210		7.6		(151) 79†	
IIb	1240		1218		30.5		(233) 126†

* Calcium salt of α-glycerophosphate (Bios), 97 per cent α.

† Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

Results

When we recently observed (3) a high percentage increase of the rate of synthesis of glycerophosphate by the addition of phosphocreatine (250 per cent increase), of glucose-1-phosphate (130 per cent), and of fructose-

1-phosphate (100 per cent), it was surmised that this increase might result from a transphosphorylation from phosphate donors of higher phosphate energy to acceptors of lower energy. It could easily be demonstrated by use of P^{32} that this was actually the case. When the phosphate donor was labeled, the synthesized glycerophosphate or fructose phosphate became highly radioactive, while the specific activity of the inorganic phosphate resulting from a partial split of the phosphate donor remained very low. In the other case, when the inorganic phosphate was labeled with P^{32}, the synthesized glycerophosphate or glucose phosphate in the presence of unlabeled phosphopyruvate had a very low radioactivity for the first 15 minutes, proving that most of the esterified phosphate did not come from the inorganic phosphate but from the phosphate donor.

TABLE VII

A. Phosphate Transfer during Enzymatic Hydrolysis of Glycerophosphate in Presence of P^{32}-Phosphocreatine

Experiment No.	Enzyme units per ml.	Initial molar concentration				Glycerophosphate split	Phosphocreatine split
		Glycerol	Inorganic P	Phosphocreatine	Glycerophosphate*	*per cent*	*per cent*
Ia	219	1.53	0.007	0	1.40	70.5	
Ib	219	1.53	0.007	0.022	1.40	35.4	74.0
Ic	219	0	0.007	0.022	0		94.0
IIa	219	1.53	0.024	0	1.40	48.5	
IIb	219	1.53	0.024	0.022	1.40	18.5	56.0
IIc	219	0	0.024	0.022	0		75.0
IId	219	0	0.024	0.022	1.40	70.5	65.0

B. Specific Activities of (b) and (d) Samples

Counts per minute per microgram of P.

Experiment No.	Phosphate species			
	Phosphocreatine		Inorganic P	Glycerophosphate
	0 min.	15 min.	15 min.	15 min.
Ib	1195	1200	449	(557) 527†
IIb	1250	1293	280	(443) 378†
IId	1250	1260	380	(93) 0.71†

* Sodium salt of glycerophosphate (Heyden), 60 per cent α; 40 per cent β.

† Corrected for non-labile, radioactive organic P impurities in the phosphocreatine.

In order to calculate the relative effectiveness of the transphosphorylation compared with the synthesis of the ester from inorganic phosphate, the acceleration of synthesis in the presence of the phosphate donor must be taken into account. This acceleration, as can be seen from the *B* sections of the tables, is quite different for the various combinations: In the early period of the incubations (15 minutes) it is 600 per cent from phosphopyruvate to glycerol or glucose, 200 per cent from phosphocreatine to glycerol or fructose, and 100 per cent from glucose-1-phosphate to glycerol. This increase in speed has to be compared with the specific activity of the synthesized ester in relation to the activity of the inorganic phosphate and the phosphate donor. This is very simple when the phosphate donor is unlabeled and the inorganic phosphate labeled. In the experiments with phosphopyruvate (Table V) the 15 minute values of glycerophosphate and of glucose phosphate have respective activities of 20 and 17 per cent of the activity of the inorganic phosphate. Therefore, 80 and 83 per cent, respectively, of the synthesized esters was formed by transphosphorylation. For a 600 per cent increase in speed the theoretical figure is 85.5 per cent. However, the kinetic exchange between the newly formed ester and the inorganic phosphate must tend to decrease this value the longer the incubation lasts. We, therefore, conclude from these experiments that, in this case, the acceleration stems wholly from transphosphorylation.

When the phosphate donor contained the labeled phosphate, a correction was necessary for the non-labile impurities. These were negligible in the glucose-1-phosphate, but amounted to 3 to 4 per cent in the phosphocreatine. These impurities had the same specific activity as the phosphocreatine and bore more weight the smaller the amount of synthesized ester. The values measured directly were corrected accordingly (values indicated in the tables). For example, in Experiment I of Table I, the corrected value for glycerophosphate gave 1487 counts[3] compared with 2210 counts for phosphocreatine and 15 for inorganic phosphate (15 minutes). The acceleration of the synthesis was 218 per cent. Therefore, we expect 31.5 per cent synthesized from inorganic phosphate and 68.5 per cent from phosphocreatine. Indeed, 1487 is 67 per cent of 2210. Here, again, excess synthesis over the control is wholly explained by transphosphorylation.

[3] This value is calculated from the measured non-labile impurity remaining at the end of incubation and from the specific activity of the phosphocreatine species for the same time period. This presupposes that the activity of the non-labile impurity exchanges in the same way as that of the phosphocreatine itself. Because the possible exchange of the P^{32} in the impurity could not be established, this calculation under the circumstances seemed to be based upon the simplest assumption.

If we apply the same reasoning to the transphosphorylation from phosphocreatine to fructose and also to that from glucose-1-phosphate to glycerol, we find the transphosphorylation to be less complete. The acceleration in Experiment II of Table III was 175 per cent; 64 per cent should, therefore, be formed by transphosphorylation. But the value was 607/2145 or 28 per cent; similar results were obtained in the other experiments. The acceleration with glucose-1-phosphate was 100 per cent (Table IV). Instead of an expected activity of the synthesized ester of 50 per cent that of glucose-1-phosphate we found only 29 per cent. It seems, therefore, that in the cases in which the acceleration is smaller, it is only in part, but not wholly, explained by transphosphorylation.

As the results of Tables VI and VII indicate, the exchange of P^{32} from phosphocreatine to glycerophosphate can be demonstrated in a state of near equilibrium and, to a smaller extent, even during dephosphorylation of glycerophosphate. In the former case, the activity of the remaining glycerophosphate increases with time, but less than would be expected if the rate of exchange were uniform. With dephosphorylation of glycerophosphate the excess activity over that of the inorganic phosphate is small because the latter increases rapidly with time as a result of the splitting of the active phosphocreatine. It can, however, still be seen with as low a concentration of inorganic phosphate as 0.007 M (Table VII, Experiment I). In the absence of free glycerol the glycerophosphate was split, practically without any reverse reaction, and did not take up any measurable activity, not even from the inorganic phosphate (Table VII, Experiment IId).

DISCUSSION

According to the experiments described in the foregoing pages the transfer of phosphate by means of phosphatase seems to be a common phenomenon. Although we have not investigated types of phosphatases other than the alkaline intestinal phosphatase, we would expect this phenomenon to be general. The fact that in the balance the transfer goes always from higher energy phosphate to lower energy phosphate is to be expected from thermodynamic considerations, if all components are in free exchange with one another. Kinetically, the opposite transfer also occurs, but it must be relatively slow and it is observed in our experiments only by a small dilution (5 to 10 per cent) of the P^{32} in the phosphate donor during the time of incubation. This percentage dilution, however, is not far from the over-all experimental error.

The findings of Axelrod (1) that some, but not all, acid phosphatases and no alkaline phosphatase allow the esterification of certain primary aliphatic alcohols in the presence of aryl phosphates, like nitrophenyl and phenyl phosphate, must be regarded as a special and exceptional case in the light of our results. If such an accumulation of an aliphatic phosphate ester occurs in spite of the phosphatase, which would normally hinder this tendency, it shows that the phosphatases in question are not able to split readily the specific esters which have formed. For example, the equilibrium constant of methyl phosphate, calculated from the measurements of McVicar (12), is K = (methanol × phosphate)/(water × methyl phosphate) = about 1.0 at pH 8.2 and probably greater than 2.0 at pH 6. This means that with 3.5 M methanol, 0.018 M inorganic phosphate, and 47.5 M H_2O only 8×10^{-4} M methyl phosphate can form in the equilibrium at pH 6. But in the experiments of Axelrod ((2) p. 297) 1.2×10^{-2} M methyl phosphate, 15 times as much, was formed. This apparently is "trapped" because the enzyme cannot readily split it.[4] In the case of the alkaline phosphatase such accumulation is impossible and the transfer, although it occurs as well as with the citrus fruit phosphatase, is with non-isotopic components visible only as an increase in speed of synthesis before the final equilibrium distribution is obtained. This invisible transfer can be studied directly by the use of P^{32} (see (13)).

The shift of the phosphate from the higher energy level to the lower is the more rapid the larger the energy difference. From phosphopyruvate to glycerol the increase in speed is 7-fold, from phosphocreatine 3-fold, and from glucose-1-phosphate 2-fold. The respective ΔF^0 values of the phosphate bond are as follows: for phosphopyruvate, −16,000 calories; for phosphocreatine, −11,500 calories; for glucose-1-phosphate, −4800 calories; for glycerophosphate, −2200 calories. These values together with those of the P^{32}-phosphate exchange presented here show that the energy differences between the phosphate donors and acceptors determine the extent of transfer per unit of time.

Because the concentrations of phosphate and polyalcohol must be high for an actual synthesis of the phosphate esters to occur, it is uncertain whether this mechanism of transfer has a biological application. However, as the experiments of Table VII show, the transfer can be observed even against the direction of the final equilibrium and with as low a phosphate concentration as 0.007 M. In any case, it is important from a theoretical view-point. It shows that the phosphatase reacts with phosphate acceptors as well as with phosphate donors: if no acceptor is present, the phosphate becomes directly liberated as inorganic phosphate; if an acceptor is present the phosphate group is at first transferred from the higher energy to the lower energy level before being split off.

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[4] It was observed by Axelrod (*cf.* (1) p. 3) that methyl phosphate was split by citrus phosphatase 7 times more slowly than was nitrophenyl phosphate at an equivalent concentration.

We thank Mr. John W. Harris, Jr., for assistance in the counting of the radioactive samples.

SUMMARY

The increase in the speed of synthesis of biological phosphate esters in the presence of alkaline intestinal phosphatase, if phosphate compounds of higher phosphate energy are added, is caused by transphosphorylation. This is proved by the use of phosphate donors, labeled with P^{32} and unlabeled inorganic phosphate, or by using unlabeled donors and radioactive inorganic phosphate.

In the system P^{32}-phosphocreatine and glycerol, 67 per cent of the total synthesis is caused by transphosphorylation, while from the increase in speed 68.5 per cent would be expected. In the system phosphopyruvate and glycerol or glucose and labeled inorganic phosphate, 80 and 83 per cent, respectively, of the synthesis is caused by transphosphorylation, while from the speed 85.5 per cent is expected.

In the systems phosphocreatine and fructose and P^{32}-glucose-1-phosphate and glycerol only half of the increase in speed can be explained by transphosphorylation, according to the specific activities of the reactants and products.

This transphosphorylation can be demonstrated also when glycerophosphate, glycerol, and phosphate are in equilibrium in the presence of labeled phosphocreatine and even if glycerophosphate, in the presence of glycerol, breaks down, owing to phosphate concentrations below the equilibrium value.

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STUDIES ON ADENOSINETRIPHOSPHATASE OF MUSCLE*

III. THE LIPOPROTEIN NATURE OF THE MAGNESIUM-ACTIVATED ADENOSINETRIPHOSPHATASE

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In the initial work (1) on the partial isolation and properties of a new magnesium-activated ATPase[1] of muscle, it was observed that the enzyme preparations contained a large amount of organic phosphorus, most of which was lipide phosphorus. The relatively small amount of nucleic acid phosphorus could be removed with ribonuclease without impairing the activity of the ATPase. Attempts to separate the enzyme from the lipide material were unsuccessful. The present report is the result of continued attempts to purify the ATPase by separation of the protein and lipide components. These experiments have produced substantial evidence that the association between lipide and protein is essential for the characteristic ATPase activity.

Methods

Preparation of Mg-ATPase—The procedure used in this work was a modification of that reported previously (1). The composition of the extracting solution was as follows: 0.1 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3, and 0.001 M KCN. The muscle was first run through a meat chopper, then mixed with 5 volumes of the extracting solution, and agitated in a Waring blendor for 1 minute. After standing about 30 minutes, the suspension was centrifuged at 4500 r.p.m. for 15 minutes. The residue was suspended in 5 volumes of the extracting solution and centrifuged as before. The procedure was then repeated a second time. The three extracts were combined, diluted with an equal volume of 0.001 M KCN, centrifuged to remove myosin, and the ATPase precipitated with

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[1] ATP = adenosine triphosphate; ATPase = adenosinetriphosphatase; Mg-ATPase = magnesium-activated adenosinetriphosphatase.

$(NH_4)_2SO_4$ at 35 per cent saturation. After centrifuging, the precipitate was washed once with 35 per cent saturated $(NH_4)_2SO_4$ in order to pack it more firmly and then redissolved in a 1:1 dilution of the extracting solution. The $(NH_4)_2SO_4$ precipitation was repeated once. The precipitate was dissolved in a mixture of 0.06 M histidine, 0.2 M KCl, and 0.001 M KCN, the volume being about equal to the original weight of muscle. The manipulations up to this point were carried out as a continuous operation. After clarifying the final solution of the ATPase by centrifuging at 6000 × *g* for 20 minutes, the ATPase was sedimented by centrifuging at 18,000 × *g* for 2 to 3 hours. The precipitate was dissolved in about one-fifth the original volume and in the same histidine-KCl-KCN buffer.

Since large amounts of the enzyme were required for these experiments, rabbit muscle was used. The preparations from this source have never been as satisfactory with regard to both yield and purity as those obtained from rat muscle. Q_P values[2] for the preparations used in the work reported here varied from 5000 to 8000. The procedure given above for the preparation of the Mg-ATPase is somewhat more convenient for the quantities of muscle used (about 300 gm.), but the yield is only 80 per cent of that obtained by the former method.

Preparation of Clostridium welchii Lecithinase—Toxic filtrates of *C. welchii* type A cultures were furnished by the Lederle Laboratories.[3] The solutions were first dialyzed against distilled water. The enzyme was then adsorbed on calcium phosphate at pH 6.5 in the presence of 25 per cent acetone, centrifuged, and eluted with 15 to 20 per cent saturated $(NH_4)_2SO_4$, according to the procedure of van Heyningen (2). This procedure was repeated once, thereby bringing an initial volume of 1 liter down to about 15 ml. After dialysis the preparation was dried from the frozen state. A well dialyzed preparation had an activity of about 15 to 20 units per mg., a unit being defined as the amount of enzyme producing 1 μM of phosphorylcholine in 10 minutes at pH 7.3 to 7.4 and 38° in the presence of about 200 γ of lecithin P in a total volume of 1.3 ml.

It has been demonstrated by Macfarlane and Knight (3) that this lecithinase acts upon lecithin to produce phosphorylcholine and a diglyceride. It has been further demonstrated by Zamecnik, Brewster, and Lipmann (4) and Macfarlane (5) that the lecithinase will not act on lysolecithin, glycerophosphorylcholine, or "cephalin," but attacks only lecithin and sphingomyelin. Following the suggestion of Zamecnik *et al.* (4), we dissolved our lecithinase preparations in 1 per cent serum albumin (crystalline bovine serum albumin obtained from Armour and Company) in order to stabilize the enzyme in solution.

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[2] Q_P = microliters of H_3PO_4 split off per mg. of protein per hour at 38°.

[3] We thank Dr. I. S. Danielson of the Lederle Laboratories Division, American Cyanamid Company, for supplying this material.

Determination of ATPase Activity—The procedure was identical with that used previously (1). The system consisted of 0.1 ml. of 0.15 M $MgCl_2$, 1.0 ml. of histidine buffer (0.1 M, pH 6.8 to 6.9 at 38°), 0.5 ml. of ATP solution (approximately 660 γ of 7 minute-hydrolyzable P per ml.), 0.2 ml. of the ATPase solution, and H_2O to a total volume of 3.0 ml. The time was 5 minutes and the temperature 38°. The reaction was stopped by addition of 3.0 ml. of 5 per cent trichloroacetic acid. After centrifuging, 2.0 ml. aliquots were analyzed for inorganic phosphorus.

Determination of Lecithinase Activity—Lecithin was prepared from eggs by the cadmium salt procedure of Levene and Rolf (6). With these preparations it was observed that satisfactory emulsions could only be obtained by sonic vibration (9000 cycles). With the Raytheon vibrator[4] 20 minutes treatment at room temperature was sufficient to produce a slightly opalescent emulsion of 2 to 3 per cent lecithin. The system employed for determination of activity consisted of 0.3 ml. of 0.1 M histidine (pH 7.3 to 7.4 at 38°), 0.1 ml. of 0.05 or 0.075 M $CaCl_2$, 0.3 ml. of 2 per cent lecithin (approximately 200 γ of P), 0.2 ml. of the lecithinase in 1 per cent serum albumin, and water to a total volume of 1.3 ml. The time of incubation was 10 minutes and the temperature 38°. Two different procedures were used in stopping the reaction and in making the analysis for the product. The first of these involved the use of trichloroacetic acid, centrifuging, filtering, and determining phosphorus on an incinerated aliquot of the filtrate. The second consisted of heat denaturation, centrifugation, filtration, digestion of an aliquot of the filtrate with alkaline phosphatase to hydrolyze the phosphorylcholine, and determining inorganic P after the phosphatase treatment. The preparation of alkaline phosphatase from intestinal mucosa followed the procedure of Schmidt and Thannhauser (7).

In the first method, 0.2 ml. of 40 per cent trichloroacetic acid was added to the incubation mixture. After centrifuging and filtering, 0.5 ml. of the filtrate was incinerated and the inorganic phosphorus determined. In the second procedure, the incubation tubes were placed in a boiling water bath for 5 minutes (no difference was observed with longer periods of time), then centrifuged, and the contents filtered. A 0.6 ml. aliquot of the filtrate was removed and to it was added 0.4 ml. of 0.05 M sodium borate containing about 50 units of alkaline phosphatase as defined by Schmidt and Thannhauser (7).

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[4] Model 9KC magnetostriction oscillator, Raytheon Manufacturing Company, Boston, Massachusetts.

After incubation with the phosphatase for 8 hours at 38°, 0.2 ml. of 40 per cent trichloroacetic acid was added, the mixture centrifuged, and 1.0 ml. of the supernatant analyzed for inorganic phosphate.

The two procedures gave comparable results, those by the phosphatase method being somewhat more consistent. A comparison of the two methods is given in Table I.

Procedures Employed in Determining Effect of Lecithinase Action on ATPase Activity—These experiments consisted of three parts: (1) incubation of the ATPase with the lecithinase, (2) determination of ATPase activity of the mixture at the end of the incubation, and (3) denaturation of the remainder of the mixture of ATPase and lecithinase and determination of the amount of acid-soluble phosphorus formed from the ATPase by lecithinase.

The experimental arrangement consisted of mixing 1.0 ml. of the concentrated ATPase solution containing 150 to 200 γ of organic P per ml., 0.1 ml. of 0.075 M $CaCl_2$, and 0.2 ml. of the lecithinase solution. Amounts of lecithinase varied from 0.46 to 45.0 unit and the time of incubation from 10 to 60 minutes. At the end of the incubation period 0.3 ml. of the mixture was added to 4.7 ml. of cold buffer (0.06 M histidine-0.2 M KCl-0.001 M KCN). After mixing, the ATPase activity of a 0.2 ml. aliquot of this diluted material was determined. The remainder of the original ATPase-lecithinase solution was inactivated and analyzed for soluble phosphorus; both the procedures given above for the determination of lecithinase activity were used.

TABLE I

Comparison of Incineration and Phosphatase Methods for Determining Lecithinase Activity

Volume of lecithinase solution	Incineration procedure	Phosphatase procedure
ml.	γ *P*	γ *P*
0.2	29.9	34.2
0.4	69.3	65.1

It was observed previously (1) that Ca inhibits the Mg-activated ATPase. However, considering the dilution of the Ca in these experiments, no significant depression of activity was anticipated. Nevertheless, to cover any instability of the ATPase during incubation at 38° for prolonged time periods under these circumstances, controls (lecithinase solution replaced by 1 per cent serum albumin) were run for each time period used. The controls always gave a small amount of soluble phosphorus (about 7 per cent of the total P) regardless of the procedure used for inactivation and analysis. In addition, the lecithinase solutions always contained some phosphorus and corrections for this were applied.

Determination of Phospholipide Fractions of ATPase Preparations before and after Action of Lecithinase—The general plan of these experiments was to inactivate the ATPase with a large amount of lecithinase, dialyze the resulting solution to remove phosphorylcholine that had been formed, and determine the composition of the remaining phospholipide. This would then be compared with the analysis of an untreated control.

For these studies 5.0 ml. of the ATPase were mixed with 0.5 ml. of 0.075 M $CaCl_2$ and with about 200 units of the lecithinase dissolved in 1.0 ml. of 1 per cent serum albumin. A control with 1 per cent serum albumin substituted for the lecithinase solution was also prepared. These solutions were incubated for 60 minutes at 38° and then transferred to dialyzing tubes and dialyzed against four or five changes of histidine buffer for 16 hours in a rocking dialyzer. After dialysis, aliquots of the solutions were removed for determination of ATPase activity and for total phosphorus content. The remainder of the dialyzed solutions was lyophilized, with final drying over P_2O_5 under a vacuum. 10 mg. portions of the dried products were removed for determination of total phosphorus, and, after weighing, the remainder was extracted with a 1:1 mixture of methanol-chloroform for 12 to 20 hours.

Analyses of the distribution of lipide phosphorus were conducted according to the procedure of Hack (8). The lipide extracts were transferred to 25 ml. volumetric flasks and made up to volume with the methanol-chloroform mixture. 1.0 ml. aliquots were removed for determination of total lipide P. Of the remaining methanol-chloroform solutions, 10 ml. of the control or 20 ml. of the lecithinase-treated material were evaporated to dryness under a stream of nitrogen at 65°. The residues were then emulsified with 5.0 ml. of 1.0 N NaOH and incubated at 38° for 16 hours. At the end of this time, the solutions were cooled and to 1.0 ml. aliquots were added 1.0 ml. of 1.5 N HCl and 3.0 ml. of 10 per cent trichloroacetic acid. These were allowed to stand for 1 hour and then centrifuged and filtered. 2.0 ml. aliquots of the filtrates were then incinerated for determination of the monoaminophosphatide P. Sphingomyelin, since it is not affected by the alkali treatment, is found in the precipitate removed by centrifugation and filtration. The diaminophosphatide (sphingomyelin) P is the difference between the monoaminophosphatide P and total lipide P.

The amount of lecithin in the monoaminophosphatide fraction was obtained by choline analysis. 3.0 ml. of the alkaline solution were treated with 1.0 ml. of 4.5 N HCl. After standing for 60 minutes, the suspension was filtered through a thin layer of paper pulp with suction and the filter washed twice with 0.5 ml. portions of 1.5 N HCl. To the combined filtrates were then added 2.0 ml. of a freshly prepared saturated solution of ammonium reineckate in 0.5 N HCl. This was allowed to stand for 30 minutes at room temperature. The crystals of choline reineckate were then collected on a Pyrex M filter and washed twice with 1.0 ml. portions of ethanol. After drying, the salt was dissolved in 3.0 ml. of acetone and the optical density determined in the Beckman spectrophotometer at 526 mμ. A standard choline curve was obtained for amounts of choline ranging from 1 to 6 μM in 5.0 ml.

Although the lecithinase preparations contained phosphate, this was all inorganic and, therefore, did not contribute to any of the fractions in these analyses.

Results

Inactivation of ATPase by Lecithinase—Fig. 1 presents the results of five experiments (Curve I) relating inactivation of the ATPase to lecithinase action. One of these (Curve II) is presented separately to indicate the uniform relationship within a single experiment. The curve in both cases is the same and was fitted to the data of the experiment which is represented separately. In these five experiments five ATPase preparations and two lecithinase preparations were employed. The characteristics of the curves are in all cases the same: an initial non-linear portion up to about 20 per cent inactivation and a linear relationship between inactivation and hydrolysis thereafter. Within the error of the experiment, the slopes were identical and the extrapolated maxima fell between 60 and 70 per cent hydrolysis of the total phosphorus. The values on the abscissa have been represented as per cent of the total P split off by the lecithinase, since complete data on the distribution of phosphorus were not obtained for all preparations of the ATPase. However, information on some of the preparations, such as that presented in Table IV, indicate that these extrapolated maxima represent the per cent of the total P present as lecithin and sphingomyelin. Complete inactivation of the ATPase was never attained, the maximal values lying between 75 and 90 per cent. With an incubation time of 60 minutes the maximal values were nearly obtained with about 20 units of the lecithinase; doubling the concentration of lecithinase gave only about 1 to 4 per cent increase in both inactivation and phospholipide hydrolysis.

The lecithinase preparations used in these experiments were probably not free of the other known activities of the toxic filtrates of *C. welchii*. Since the lecithinase is activated by Ca, by reducing the Ca concentration it should be possible to demonstrate whether or not the association of inactivation of the ATPase with phospholipide hydrolysis is fortuitous. Experiments of this type were performed, with and without added Ca, the system being otherwise constant (final concentration of addition, 5.7 × 10^{-3} M Ca). Some results are given in Table II. There was always some lecithinase activity in the absence of added Ca, since Ca phosphate was used in the preparation of the lecithinase and was never completely removed. Both inactivation of the ATPase and phospholipide hydrolysis were appreciably reduced in the absence of added Ca. The higher concentrations of lecithinase in Table II are in excess of that required to give maximal inactivation and hydrolysis with an optimal Ca concentration and an incubation period of 60 minutes. The lecithinase activities with and without added Ca are, therefore, not in the same proportion in all the ex-

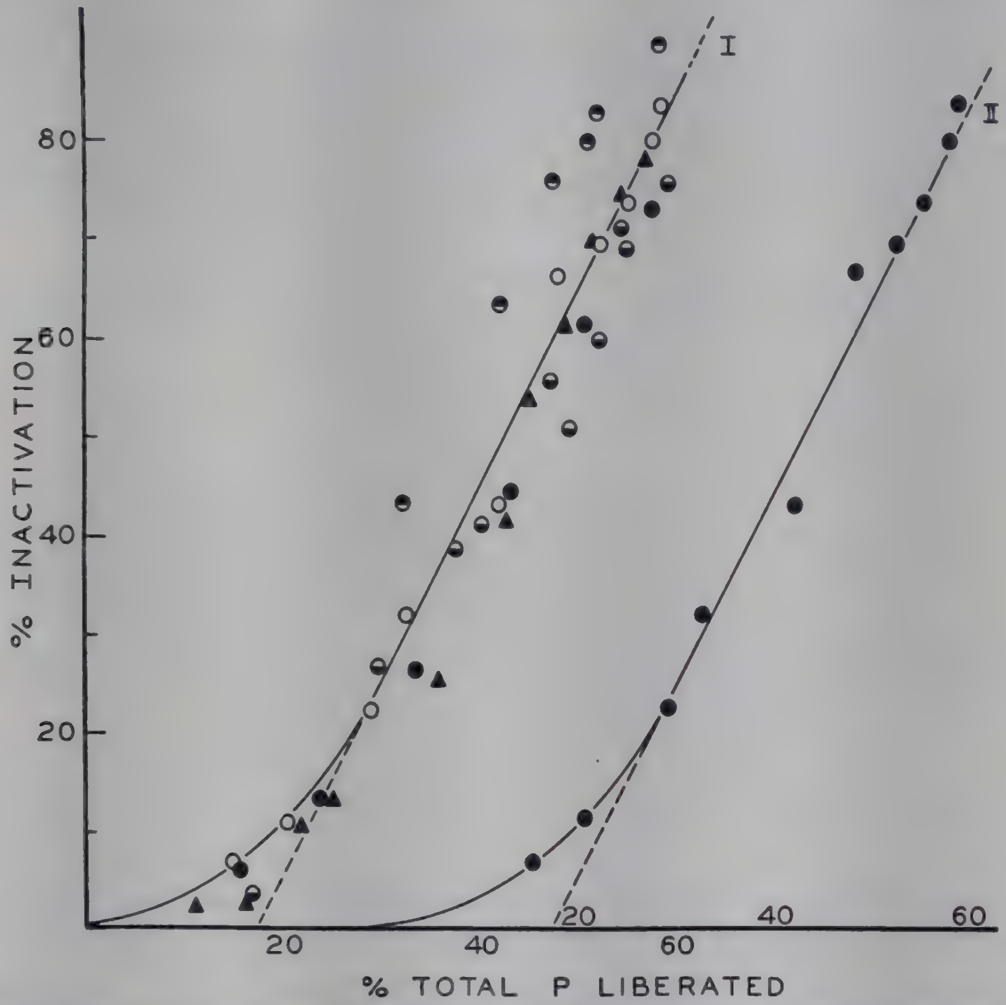

Fig. 1. Relationship between inactivation of the ATPase and formation of acid-soluble phosphorus from the ATPase preparation by lecithinase. Results for five ATPase preparations are given (Curve I). One of these (○) is presented separately to indicate the uniformity within a single experiment. The numbers on the abscissa below the line refer to the P liberated in the five experiments of Curve I; the numbers above the line refer to Curve II.

periments. Without added Ca, the inactivation is suppressed more than the phospholipide hydrolysis. No reason for this is immediately evident, though an explanation probably resides in the complex character of the phospholipide and the relative rates with which the individual components are split by the lecithinase.

Indifference of ATPase to Products of Phospholipide Hydrolysis by Lecithinase—It seemed possible that the inactivation of the ATPase by the lecithinase might be due to inhibition by the products of hydrolysis of the phospholipides rather than to destruction of a component necessary for the activity of the ATPase. To test this an amount of lecithin corresponding to 686 γ of P was treated with 2 units of lecithinase and 0.2 ml. of 0.05 M $CaCl_2$ in a total volume of 2.6 ml. When hydrolysis was nearly complete (94 per cent), 0.5 ml. of this solution, containing 132 γ of P, was mixed with 0.5 ml. of a concentrated ATPase solution containing 74 γ of organic P. Two controls were prepared, one containing the same concentrations of Ca and lecithinase, the other a similar dilution of the ATPase with histidine buffer. After standing for 30 minutes, 0.5 ml. of each solution was diluted to 5.0 ml. with the usual histidine buffer and the ATPase activity determined on a 0.2 ml. aliquot. The results are presented in Table III. It is evident that, though the hydrolyzed lecithin constituted about 70 per cent of the total phospholipide (about 80 per cent of the ATPase phosphorus being lipide P), no inhibition of the ATPase occurred.

Table II

Influence of Ca on Inactivation of Mg-ATPase and on Hydrolysis of Its Phospholipide by C. welchii Lecithinase

Time, 60 minutes; temperature, 38°.

ATPase preparation No.	Lecithinase per 1.0 ml. ATPase solution	Ca added		No Ca added	
		Inactivation	Hydrolysis	Inactivation	Hydrolysis
	units	*per cent*	*per cent total P*	*per cent*	*per cent total P*
9	1.3 (Preparation II)	26.7	33.2	0.0	1.0
9	17.0 (Preparation II)	73.7	57.5	8.0	26.8
10	26.0 (Preparation III)	83.2	51.9	18.8	32.9

Composition of Phospholipide of ATPase Preparations and Changes That Occur in It through Action of Lecithinase—The results of one experiment conducted according to the procedure given earlier for determining lipide P distribution are presented in Table IV. In this experiment 90 per cent of the ATPase activity was lost as a result of the lecithinase treatment. As a check on the effectiveness of the dialysis employed in this analysis, a parallel experiment was carried out on a smaller scale with the same material but with immediate analysis at the end of the incubation period. In this case, the amount of phospholipide split was determined by the phosphatase procedure. Identical values were obtained for loss of activity and change in total bound P in the two experiments.

TABLE III

Indifference of Mg-ATPase to Products of Phospholipide Hydrolysis by C. welchii Lecithinase

Hydrolyzed lecithin	Lecithinase	ATPase activity in 5 min.
		γ *P*
−	−	58.2
−	+	57.6
+	+	57.6

TABLE IV

Distribution of Organic P in ATPase before and after Treatment with Lecithinase

The results are expressed in micromoles of P per ml.

	Control ATPase solution (1)	Lecithinase-treated ATPase solution (2)	Difference, ATPase solution (1) − (2)
Total P	5.05	1.93	3.12
" lipide P	4.07	1.12	2.95
Monoaminophosphatide P	3.65	0.98	2.67
Choline (lecithin)	2.69	0.06	2.63
Sphingomyelin P	0.42	0.14	0.28

Within the probable error involved in the analyses presented in Table IV, the change in total P is accounted for by the change in lipide P. There appears to be no contribution from the cephalin fraction (monoaminophosphatide P minus lecithin P) and the change in lipide P is equal to the sum of the changes in lecithin (choline) and sphingomyelin which together comprise the "choline phosphatide fraction."

DISCUSSION

Except for the initial non-linear portion of the curves in Fig. 1, inactivation of the ATPase and hydrolysis of phospholipide exhibited strictly parallel courses. Maximal values appear to be a characteristic of the individual ATPase preparation. These maxima were not significantly increased by prolonging the time of incubation and increasing the amount of lecithinase. Inhibition of the lecithinase by the cephalin fraction when hydrolysis of the choline-containing phosphatides has approached completion probably accounts for the fact that complete inactivation of the ATPase was never attained. Zamecnik *et al.* (4) have demonstrated that "cephalin" will inhibit the lecithinase and by examining Table IV it is observed that, whereas the initial concentrations of the "cephalin" and choline phosphatide (lecithin + sphingomyelin) fractions were 0.96 and 3.11 μM per ml. respectively, at the end of the experiment they are 0.92 and 0.20 μM. In addition, the hydrolyzable substrate at the end of the experiment is primarily sphingomyelin which, as demonstrated by Macfarlane (5), is attacked at a slower rate than lecithin. Thus the changing relative concentrations of the phospholipide components have combined with the actual change in substrate concentration, in any experiment in which either time or lecithinase concentration was varied, to slow down and finally halt phospholipide hydrolysis and with it the inactivation of the ATPase.

The dependence of inactivation of the ATPase on phospholipide hydrolysis is further substantiated by the control exerted by Ca concentration over both phenomena.

With the demonstration that the products of lecithinase action do not suppress the ATPase activity, the conclusion seems justified that a phospholipide component of the partially purified Mg-ATPase of muscle is essential for the characteristic enzymatic activity of these preparations.

The ATPase preparations analyzed in this investigation contained all three of the general classes of phospholipides and of these both lecithin and sphingomyelin were split by the lecithinase; therefore, we can only state that the essential component is a choline-containing phospholipide.

SUMMARY

The inactivation of partially purified preparations of the magnesium-activated ATPase of muscle by *Clostridium welchii* lecithinase has been studied.

It has been found that the inactivation of the ATPase depends on the lecithinase activity and that, over the upper 80 per cent of the range, the splitting of phospholipide and inactivation are proportional.

It is concluded that a choline-containing component of the phospholipides present in the ATPase preparation is essential for the characteristic enzymatic activity.

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CORRECTION

On page 90, line 11, Vol. 181, No. 1, November, 1949, read *113–115°* for *213–215°*.

On page 764, legend to Fig. 3, Vol. 181, No. 2, December, 1949, read *3.375* for *3375* and *2.85* for *285*.

A COLORIMETRIC METHOD FOR THE DETERMINATION OF CHYMOTRYPSIN ACTIVITY*

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The L isomers of esters, amides, and hydrazides of the general formula $RCONHCHR_1COR_2$ where $R = CH_3—$, $C_6H_5—$, etc., $R_1 = C_6H_5CH_2—$, p-$HO—C_6H_4CH_2—$, etc., and $R_2 = —OCH_3$, $—NH_2$, or $—NHNH_2$, are known to be hydrolyzed by chymotrypsin (1–6). It has now been found that the corresponding hydroxamides, $RCONHCHR_1CONHOH$, where $R_1 = C_6H_5CH_2—$, are also hydrolyzed by this enzyme (Table I). Aside from the obvious usefulness of the above hydroxamides in the further definition of chymotrypsin activity we wish to point out that these latter substrates provide the basis for a simple, sensitive, and rapid colorimetric method for the determination of chymotrypsin activity which can be extended to a number of other proteolytic enzymes.

It is well known that acylhydroxamides, *i.e.* hydroxamic acids, react with ferric ion, in acidic solutions, to give characteristic deep red colored coordination compounds (7) and it has been found that the above reaction can be used in a quantitative way to determine the rate of disappearance of hydroxamides of the general formula, $RCONHCHR_1CONHOH$, when these latter compounds are being hydrolyzed by a proteolytic enzyme, in this case chymotrypsin. In practice an aliquot portion of the enzymatic reaction mixture is added to a standard acidic aqueous-methanol solution of ferric chloride, the intensity of the color is observed in a photoelectric colorimeter, and the amount of hydroxamic acid present is estimated from a previously determined calibration curve.

In addition to their offering the basis for a colorimetric procedure the hydroxamides of the general formula $RCONHCHR_1CONHOH$ possess other characteristics that make them desirable substrates. For compounds of the type $RCONHCHR_1COR_2$, where $R = CH_3—$ or $C_6H_5—$ and $R_1 = C_6H_5CH_2—$, the rate of hydrolysis, by chymotrypsin, of the corresponding esters, amides, and hydroxamides, *i.e* $R_2 = —OCH_3$, $—NH_2$, and $—NHOH$ when R and R_1 are invariant, is in the order ester > hydroxamide >

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‡ Contribution No. 1348.

amide (4, 8). Thus the hydroxamides are hydrolyzed sufficiently rapidly to permit their use with relatively low enzyme concentrations and to allow the attainment of saturation of the enzyme at relatively low substrate concentrations. These factors, coupled with the greater sensitivity of the colorimetric method, make it possible to study the hydrolytic reaction at initial substrate concentrations of less than 2.5 micromoles per ml. with a greater precision than is practicable with titrimetric methods. A further desirable characteristic of the hydroxamide type of substrate is based upon the fact that these compounds are acids and, even though their acid strength is not great (7), it is possible to use the corresponding salts with those enzymes whose pH optima are greater than 7 in the event that the hydroxamides *per se* are relatively insoluble in water. It should be pointed out that if this latter procedure is adopted, and particularly if an alkali metal salt is used, care must be taken to provide a system of adequate buffering capacity.

TABLE I

*Hydrolysis of Acetyl- and Benzoyl-L-phenylalaninhydroxamides by Chymotrypsin**

Substrate	S_0†	E_0‡	Buffer concentration§	Analytical method	Per cent hydrolysis in		
					20 min.	40 min.	60 min.
Acetyl-DL-	5	0.15	0.04	A	34	53	69
"	5	0.15	0.04	B	35	52	67
"	2.5	0.15	0.02	A	40	69	84
"	2.5	0.15	0.02	B	40	70	82
Benzoyl-DL-	2.5‖	0.15	0.02	A	*Ca.* 80% in 7 min.		
"	2.5	0.03	0.02	"	58	76	80

* At 25° and pH 7.9 (tris(hydroxymethyl)aminomethane-hydrochloric acid buffer).

† Initial concentration of L isomer in micromoles per ml. of reaction mixture.

‡ Initial enzyme concentration in mg. of protein nitrogen per ml. of reaction mixture.

§ Molar concentration of tris(hydroxymethyl)aminomethane in reaction mixture.

‖ As sodium salt.

The advantages of the above colorimetric method for the determination of proteolytic activity over other colorimetric methods (9, 10) are obvious when it is realized that the former method is based upon the use of specific substrates of known structure, whereas the latter are dependent upon relatively non-specific substrates of unknown structure.

In the present study the DL-hydroxamides have been used as substrates. In view of the fact that it is known that in other cases the D isomer can competitively inhibit the hydrolysis of the L isomer and that this effect is maximal at apparent enzyme saturation (11), it is recommended that in the application of the above colorimetric method the pure L isomer be used as a substrate until sufficient data are available to evaluate the magnitude of the various rate and inhibition constants. Such studies are now in progress.

EXPERIMENTAL[1]

Acetyl-DL-phenylalaninhydroxamide—To a solution of 12.5 gm. of 2-methyl-4-benzyl-5-oxazolone, b.p. 115–116° at 1 mm. (12) in 15 ml. of anhydrous ether, were added, with vigorous shaking and cooling in an ice-salt bath, 65.5 ml. of a 5 per cent anhydrous methanol solution of hydroxylamine. The reaction mixture was allowed to stand overnight at room temperature, filtered, the filtrate evaporated to dryness, the syrupy residue extracted with four 225 ml. portions of hot ethyl acetate, and the filtered ethyl acetate extract concentrated *in vacuo* to 150 ml. After standing for 20 hours, the last three at 0°, the crystalline precipitate was collected and dried to give 8.17 gm. of acetyl-DL-phenylalaninhydroxamide, m.p. 120–125°, with softening at 110°. After two recrystallizations from ethyl acetate, the product melted at 131–132° with decomposition.

Analysis—$C_{11}H_{14}O_3N_2$ (222). Calculated, C 59.4, H 6.3, N 12.6
Found, " 59.2, " 6.3, " 12.6

Benzoyl-DL-phenylalaninhydroxamide—To a solution of 14 gm. of benzoyl-DL-phenylalanine methyl ester in 50 ml. of anhydrous methanol were added 24.5 ml. of a 10 per cent methanol solution of hydroxylamine. The solution was cooled to 0°, 49.4 ml. of N methanolic sodium methoxide were added, and the reaction mixture allowed to stand at room temperature for 48 hours. The precipitated solid was dissolved by warming the solution to 40°. The solution was cooled to 0°, acidified to Congo red with 6 N hydrochloric acid, evaporated to dryness *in vacuo*, and the residue dried over solid sodium hydroxide and extracted with a total of 700 ml. of hot ethyl acetate. The extract was cooled, and the crystalline precipitate collected and dried to give 10.8 gm. of benzoyl-DL-phenylalaninhydroxamide, m.p. 157–158° with decomposition. A second crop of 1.2 gm., m.p. 156–157° with decomposition, was obtained from the mother liquor. A portion of the above product was recrystallized from *n*-butanol to give the hydroxamide, m.p. 158–159° with decomposition and with preliminary softening at 152°.

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[1] All the melting points are corrected.

Analysis—$C_{16}H_{16}O_3N_2$ (284). Calculated, C 67.6, H 5.7, N 9.9
Found, " 67.7, " 5.6, " 9.8

Method A—A 1.0 ml. aliquot of the enzymatic digest was added to 1.0 ml. of 36 per cent aqueous formaldehyde, previously adjusted to pH 7.0, and the solution titrated potentiometrically with 0.01 N aqueous sodium hydroxide (13).

Method B—A standard ferric chloride solution was prepared as follows: 0.50 gm. of reagent grade anhydrous ferric chloride was dissolved in 80 ml. of 1.0 N hydrochloric acid and the volume of the solution made up to 100 ml. with absolute methanol. A 1.0 ml. aliquot of the enzymatic digest was added to 5.0 ml. of absolute methanol and 1.0 ml. of standard ferric chloride solution, contained in a calibrated colorimeter tube, and the volume of the solution made up to 10.0 ml. by the addition of water. The contents of the tube were shaken and the intensity of the color determined with a Klett-Summerson photoelectric colorimeter equipped with a green filter. Solutions containing varying amounts of substrate in either 0.02 M or 0.04 M tris(hydroxymethyl)aminomethane-hydrochloric acid buffer were used for the preparation of a standard calibration curve. The values observed for the two buffer concentrations were identical and it was found that for concentrations of hydroxamide between the limits of 1 and 10 micromoles per ml. of original solution the colorimeter readings were directly proportional to the concentration of hydroxamide. The presence of enzyme in concentrations up to 0.15 mg. of protein nitrogen per ml. of original solution had no demonstrable effect upon the intensity of the color at any given hydroxamide concentration. However, at high concentrations of hydroxamide it was observed that the intensity of the color slowly faded on standing and because of this phenomenon the time interval between the time of mixing and the time of reading was standardized at 90 seconds. The experimental conditions specified for the color development were selected with regard to their suitability for the attainment of the following objectives; *i.e.*, immediate cessation of enzymatic activity, avoidance of the precipitation of substrate or hydrolysis products, and the development of maximal color intensity.

Enzyme Experiments—The chymotrypsin was an Armour preparation and the data obtained are given in Table I. The conventional controls, *i.e.* substrate alone and enzyme alone, were performed coincidentally with each separate experiment.

SUMMARY

It has been observed that the acetyl- and benzoyl-L-phenylalaninhydroxamides are hydrolyzed by chymotrypsin, and a colorimetric method for the determination of chymotrypsin activity based upon the use of hydroxamides as substrates has been described.

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THE NON-ENZYMATIC REDUCTION OF CYTOCHROME *c* BY PYRIDINE NUCLEOTIDES AND ITS CATALYSIS BY VARIOUS FLAVINS*

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It is now generally accepted that a large fraction of cellular oxidations is mediated by the pyridine nucleotide-flavoprotein-cytochrome-cytochrome oxidase system. Since the isolation of the first flavoprotein by Warburg and Christian (1), several flavoenzymes have been discovered. Although some of these are concerned with the direct oxidation of various metabolites (*e.g.*, the amino acid oxidases) and others (*e.g.*, cytochrome reductases, diaphorases) mediate electron transfer from reduced pyridine nucleotides to cytochrome and other oxidizing agents, the most important known rôle of flavoproteins in biological oxidations, according to Ball (2), remains "their participation as mediators in oxidations initiated by means of the pyridine nucleotides." While it is generally recognized that the isoalloxazine nucleus of the prosthetic group is the functional unit of flavoenzymes, to the authors' knowledge it has not been demonstrated that isoalloxazine derivatives themselves, in the absence of added proteins, are capable of catalyzing the reaction between reduced pyridine nucleotides and cytochrome *c*.

In the course of a study[1] of the inhibition of cytochrome *c* reductase (TPN, yeast) (3) by isoriboflavin we observed that in the presence of isoriboflavin or riboflavin cytochrome was rapidly reduced by $TPNH_2$ despite the absence of cytochrome reductase. We decided to investigate this reaction in detail. This paper demonstrates the reduction of cytochrome *c* by reduced DPN, TPN, and NMN and the catalytic rôle of various flavins in the reaction.

Materials and Methods

Flavins—Riboflavin was purchased from Wyeth, Inc., and was found to be a pure compound within the limits of the spectrophotometric method.

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[1] The following abbreviations are used: TPN, triphosphopyridine nucleotide; $TPNH_2$, reduced TPN; DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced DPN; NMN, nicotinamide mononucleotide; $NMNH_2$, reduced NMN; FAD, flavin-adenine dinucleotide; FM, flavin mononucleotide.

The isoriboflavin was a gift of Dr. W. Gibson of Merck and Company and was analytically pure as indicated by C, H, and N analysis. Alloxazine was obtained from the Bios Laboratories, Inc. Flavin-adenine dinucleotide (FAD) was isolated from bakers' yeast by the procedure of Warburg and Christian (4); its concentration was measured by the light absorption at 445 mμ and by the D-amino acid oxidase test (4). Flavin mononucleotide (FM) was synthesized from riboflavin (5) and its concentration was determined spectrophotometrically and by phosphorus analysis. All flavin solutions were protected from light as much as possible.

Cytochromes[2]—Two preparations of cytochrome *c* were used in most of the experiments, one from Wyeth, Inc., and the other from the Nutritional Biochemicals Corporation. Certain of the key experiments were repeated with a cytochrome of high purity (0.38 per cent Fe), prepared according to the method of Keilin and Hartree (6) and kindly provided by Dr. Ernest Bueding of the Department of Pharmacology. Purity of the cytochromes was estimated by measuring the ratio of the extinction coefficients of the oxidized and reduced forms, at 550 mμ, after treatment with ferricyanide and Na hydrosulfite, respectively (7). The ratio obtained[3] (0.329 to 0.330) was in good agreement with the figures given by Potter (7) and by Theorell (8). The concentration of cytochrome solutions was calculated from the light absorption at 550 mμ following reduction by hydrosulfite, by use of the relation $\text{CyFe}^{++} = (D/0.281) \times 10^{-8}$ mole per ml., when D = the optical density in a 1 cm. path (3). In all measurements of cytochrome reduction the Beckman spectrophotometer was used, and the amount of CyFe^{+++} present at any time was calculated from the optical density at 550 mμ by means of the equation developed by Haas *et al.* (3): $\text{CyFe}^{+++} = (1/l) \times D - 0.281 \times 10^{8} \times (\text{total CyFe})/(-0.1854 \times 10^{8})$, where l = the length of the light path in cm., D = the optical density directly read on the spectrophotometer, and cytochrome concentrations are expressed in moles per ml.

Pyridine Nucleotides—Two preparations of TPN were used, one of 37 per cent and the other of 51 per cent purity, as determined by enzymatic analysis in the *Zwischenferment* test (9). We are grateful to Dr. S. Ochoa, of New York University, for these samples of TPN. DPN (Schwarz Laboratories, Inc.) was estimated spectrophotometrically at 340 mμ following hydrosulfite or enzymatic reduction, and varied in purity from 50 to 60 per cent. Solutions of DPNH_2 were prepared by Gutcho and Stewart's modification of the hydrosulfite method (10), except that the final product was made 1.5×10^{-2} M with respect to phosphate buffer and the pH was adjusted to 7.6 to 7.9, depending on the experiment. For reasons discussed in the paper, solutions of $DPNH_2$ thus prepared were either used immediately or were preserved for short periods of time, preferably at −40°. The concentration of $DPNH_2$ solutions was calculated from the molecular extinction coefficient at 340 mμ (11), and their true $DPNH_2$ content was frequently checked by oxidation with acetaldehyde in the presence of alcohol oxidase. Some preparations of $DPNH_2$ were subjected to alcohol fractionation (11) in order to obtain a dry product free from inorganic salts; the purity was thereby raised to 78 per cent. Solutions of $NMNH_2$ were prepared from $DPNH_2$ by the action of nucleotide pyrophosphatase (12), kindly supplied by Dr. A. Kornberg of the National Institutes of Health. Complete splitting of $DPNH_2$ into $NMNH_2$ was ascertained by its lack of activity in the alcohol oxidase system, although the light absorption at 340 mμ was unaltered.

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[2] The terms ferricytochrome *c*, oxidized cytochrome *c*, and CyFe^{+++} are used interchangeably in this paper, and similarly for the reduced forms.

[3] Only the presence of colored impurities is detected by this method.

Enzymes—Zwischenferment was prepared from brewers' bottom yeast (1). The phosphoglyceraldehyde oxidase from yeast was a lyophilized preparation of the partially purified enzyme, made by Miss Aeme Higashi, of the Department of Pharmacology, by the procedure of Meyerhof and Junowicz-Kocholaty (13). Phosphoglyceraldehyde oxidase from rabbit muscle was prepared according to Cori, Slein, and Cori (14), and was used as the once recrystallized enzyme. Alcohol oxidase was isolated from yeast and assayed by the procedure of Negelein and Wulff (15); the purification was carried through the second $(NH_4)_2SO_4$ precipitation. A lyophilized preparation of cytochrome oxidase was prepared by Mr. T. Talcott of this department by an unpublished modification of the Keilin and Hartree procedure (16).

EXPERIMENTAL

Reduction of Cytochrome by Enzymatically Prepared $TPNH_2$—The protocol and results of the first experiment which led to the observation of the non-enzymatic reduction of cytochrome *c* are presented in Table I. Since this experiment was intended to measure the effect of riboflavin on cytochrome *c* reductase, the experimental conditions conform approximately with those used by Haas *et al.* for the assay of that enzyme (3). All components except riboflavin were incubated for 10 minutes at 25° to permit the reduction of TPN by the glucose-6-phosphate-*Zwischenferment* system. Riboflavin was added at zero time and readings were taken every minute against blanks which contained all additions except cytochrome *c*. It will be seen from Table I that in the complete system 20 per cent of the added cytochrome was reduced in 6 minutes at 25°. When either glucose-6-phosphate or riboflavin was omitted, no significant reduction of cytochrome occurred. In parallel experiments with the same amount of isoriboflavin

(4×10^{-7} mole per ml.) cytochrome *c* was reduced over twice as fast as in the experiment above.

There could be three possible interpretations of these findings. First, riboflavin and isoriboflavin may have catalyzed the interaction of $TPNH_2$ with ferricytochrome *c*, replacing cytochrome *c* reductase; second, an impurity in one of the reagents may have performed this function; or third, the *Zwischenferment* may have contained some split reductase protein (apoenzyme) with which the added flavins united to form an active reductase complex. The third possibility would require a considerable lack of specificity in the requirements of the prosthetic group of cytochrome reductase, but none the less was tested in two ways designed to inactivate any reductase present. First, the *Zwischenferment* system was used to accumulate $TPNH_2$ as before, with corresponding blanks lacking glucose-6-phosphate. These solutions were then cautiously acidified to pH 2.5 to 3.0 at 0° with 20 per cent CCl_3COOH and rapidly filtered in the cold. The neutralized filtrates gave negative biuret reactions and were devoid of *Zwischenferment* activity in the Haas dye test (9). The second means of inactivating the *Zwischenferment* system following accumulation of $TPNH_2$ was by heating the solutions in a boiling water bath for 2 minutes, and rapidly cooling them to room temperature. Although both of these tech-

TABLE I

Reduction of Ferricytochrome c in Presence of Riboflavin

D at 550 mμ, read *vs.* reagent blank			Time
No glucose-6-phosphate	No riboflavin	Complete system	
			min.
0.462	0.449	(0.462)	0
0.463	0.448	0.505	1
0.465	0.449	0.522	2
0.466	0.449	0.546	3
0.467	0.448	0.556	4
0.468	0.449	0.583	5
0.468	0.449	0.601	6
		3.73×10^{-8} mole per ml.	$(CyFe^{+++})$ at $t = 0$
		2.99×10^{-8} “ “ “	“ “ “ = 6 min.
		0.74×10^{-8} “ “ “	$\Delta(CyFe^{+++})$ in 6 min.

Zwischenferment, 1 ml. ≈ 0.8 mg. in 0.1 M PO_4, pH 7.3, 0.4 ml.; glucose-6-phosphate, 1 ml. ≈ 6.1 mg. of Ca salt, 0.4 ml.; TPN, 1 ml. ≈ 810 γ of 37 per cent pure substance, 0.2 ml.; PO_4 buffer, 0.1 M, pH 7.3, 0.35 ml.; $CyFe^{+++}$, 2.5×10^{-4} M, 0.45 ml.; riboflavin, 2×10^{-3} M in 5×10^{-3} M NaOH, 0.6 ml.; H_2O to 3.0 ml.; pH 7.44.

niques inactivated much of the $TPNH_2$ present, the light absorption at 340 mμ indicated enough $TPNH_2$ to permit the use of these two preparations as reducing agents for cytochrome *c* in the presence of riboflavin. With both preparations there was a rapid and continuous reduction of cytochrome until the $TPNH_2$ was exhausted. No reduction occurred with the corresponding blanks prepared without glucose-6-phosphate. These findings made it unlikely that a cytochrome reductase impurity in the *Zwischenferment* was responsible for the reduction of cytochrome in the original experiment. It should be added, however, that a preparation of *Zwischenferment* essentially free from reductase, kindly placed at our disposal by Dr. B. L. Horecker of the National Institutes of Health, gave entirely similar results.

The second possibility, that impurities in the substances used were reducing cytochrome, was next studied. Since the glucose-6-phosphate, riboflavin, and isoriboflavin were analytically pure compounds, such impurities would be most reasonably expected in the *Zwischenferment*, cytochrome, or TPN. In order to eliminate these factors in *Zwischenferment* and in TPN, it was thought advisable to establish the reduction of cytochrome by $DPNH_2$, prepared either by hydrosulfite reduction or by enzymatic reduction of DPN.

Reduction of Cytochrome by Non-Enzymatically Reduced DPN—The experiment illustrated in Fig. 1 demonstrates the oxidation of chemically reduced DPN by ferricytochrome *c*, as catalyzed by various amounts of riboflavin. The $DPNH_2$ was prepared, as described above, immediately before the experiment. A control solution of hydrosulfite in bicarbonate-carbonate buffer, but without DPN, was carried through the Gutcho and Stewart procedure and then treated with riboflavin and cytochrome. No reduction of the latter occurred, indicating the complete removal of reducing substances other than $DPNH_2$ itself by the aeration procedure. It is apparent from Fig. 1 that the chemically reduced DPN preparation, like the enzymatically reduced TPN described above, is capable of reducing ferricytochrome *c* in the presence of riboflavin, but not in its absence, and that the rate of the reaction is approximately proportional to the concentration of riboflavin. It is also apparent that the rate of reduction of cytochrome *c* at all flavin concentrations follows a second order reaction, wherein two reactants ($2CyFe^{+++}$), present at equal concentrations, determine the rate. This conclusion was arrived at by plotting ($CyFe^{+++}$), log ($CyFe^{+++}$), and $1/(CyFe^{+++})$ as a function of time. Only the last one gave a linear relationship. It may be noted that the data presented in Table I also follow a second order reaction curve. The significance and interpretation of these reaction orders will be discussed in the section on kinetics. Similar results were obtained when the $DPNH_2$ preparation was further purified and freed of inorganic salts by fractional alcohol precipitation (11).

Reaction of Enzymatically Reduced DPN with Ferricytochrome c—In order to substantiate the fact that the $DPNH_2$ itself, rather than an impurity, was the reducing agent in the foregoing experiments, DPN was reduced specifically by means of three enzymes, and the activity of the resulting products on cytochrome *c* and riboflavin was subsequently determined. The first enzyme used was alcohol oxidase from yeast.

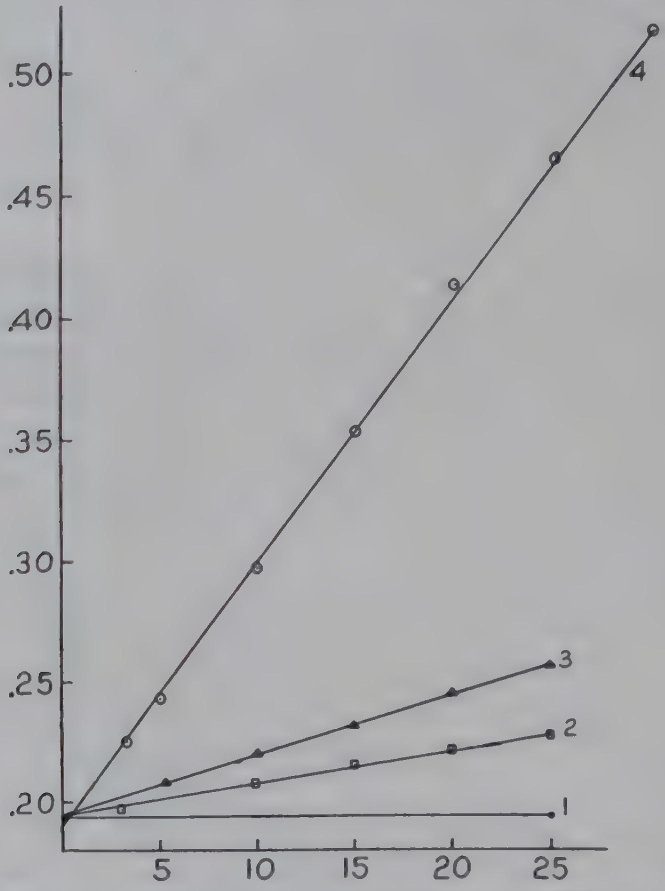

FIG. 1. The rate of reduction of ferricytochrome *c* by $DPNH_2$ at various flavin concentrations. Abscissa, time in minutes; ordinate, $1/(CyFe^{+++})$ moles per ml. $\times 10^{-8}$. Curve 1, no riboflavin; Curves 2, 3, and 4, 3.4×10^{-8}, 8.6×10^{-8}, and 34.3×10^{-8} mole per ml. of riboflavin, respectively. Each cuvette contained in a volume of 3.5 ml. 5.15×10^{-8} mole per ml. of $CyFe^{+++}$, 1 ml. of 0.1 M phosphate, pH 7.3, 6.31×10^{-8} mole per ml. of $DPNH_2$, and the indicated amount of riboflavin. Average temperature, 26.3°; readings taken at 550 mμ.

Dimedon (dimethyldihydroxyresorcinol) was used as an aldehyde fixative in the reaction, since it does not react with cytochrome or inhibit alcohol oxidase, but efficiently binds acetaldehyde. In some experiments alcohol oxidase was inactivated by means of *p*-chloromercuribenzoate, after complete reduction of the DPN, before the addition of cytochrome and riboflavin to the system. The results were the same whether this procedure was followed or the $DPNH_2$ was continuously regenerated by the active oxidase throughout the subsequent reaction with riboflavin and cytochrome.

Fig. 2 summarizes the results of a typical experiment on the reaction between ferricytochrome *c* and $DPNH_2$ produced by the action of alcohol oxidase. It is seen that no reduction took place when alcohol was omitted and the accumulation of $DPNH_2$ was thereby prevented (Curve 3); very little reaction took place without added flavin (Curve 1), while riboflavin in a final concentration of 24.2×10^{-8} mole per ml. brought about a rapid reduction of the cytochrome (Curve 2). The concentration of $CyFe^{+++}$ at given time intervals is plotted directly in Fig. 2, rather than its reciprocal since the reaction did not follow strictly a second order reaction curve.

Entirely similar results were obtained when $DPNH_2$ was produced by means of crystalline phosphoglyceraldehyde oxidase from rabbit muscle or by a highly purified preparation of this enzyme from yeast, except that the aerobic reduction of cytochrome by the $DPNH_2$ thus obtained followed a second order reaction rate.

Inasmuch as the reduction of cytochrome depended on the presence of $DPNH_2$ or $TPNH_2$ and flavin in all of the foregoing experiments, regardless of the means by which the reduced pyridine nucleotides were generated, it seemed logical to conclude that these agents themselves were necessary in this reaction. There remained two points to be tested: (*a*) whether a colored impurity in the cytochrome preparation or ferricytochrome *c* itself was the oxidizing agent; (*b*) whether an impurity present in the cytochrome in small amounts acted as a catalyst for the oxidation-reduction reaction. The first of these points was eliminated in part by the purity measurements summarized under "Materials and methods," but was further tested as described in the following section.

Reduction of Cytochrome from Various Sources; Effect of Cytochrome Oxidase—Five different cytochrome *c* preparations were tested on $DPNH_2$ as substrate under the conditions given in Fig. 1. These were products obtained from Wyeth, Inc., Sharp and Dohme, Inc., the Nutritional Biochemicals Corporation, and the Sigma Chemical Company. The fifth product was the Keilin and Hartree preparation, containing 0.38 per cent Fe. All of these cytochromes behaved in an identical manner. In addition, it should be mentioned that the oxidized cytochrome could be completely reduced, particularly under anaerobic conditions, provided that enough reduced nucleotide was present. Lastly, the experiment recorded

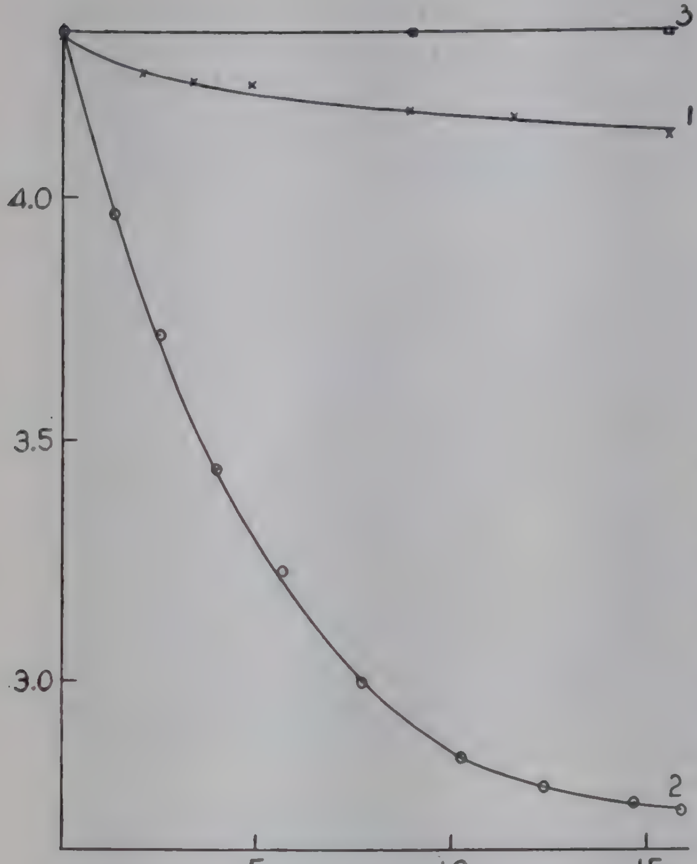

FIG. 2

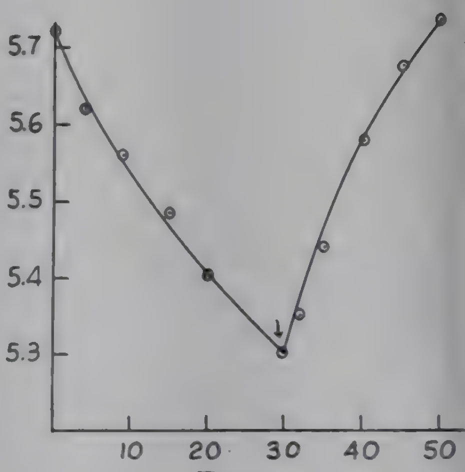

FIG. 3

FIG. 2. Reduction of cytochrome *c* with and without added riboflavin with continuous production of $DPNH_2$ by alcohol oxidase. Abscissa, time in minutes; ordinate, $CyFe^{+++}$ concentration in moles per ml. $\times 10^{-8}$. Curve 1, no flavin added; Curve 2, 24.2×10^{-8} mole per ml. of riboflavin; Curve 3, no alcohol. Two identical tubes containing 0.4 ml. ≈ 0.96 mg. of 50 per cent DPN, 1.2 ml. of 10^{-1} M pyrophosphate, pH 7.8, 0.1 ml. of 3 per cent glycine, 0.3 ml. of 5×10^{-2} M dimedon, 0.3 ml. of 3 M ethanol, and 0.1 ml. of 1:150 alcohol oxidase were incubated for 10 minutes at 30°. Tube 1 received 0.5 ml. of 2.87×10^{-4} M cytochrome + 0.4 ml. of H_2O (Curve 1). To Tube 2, 0.5 ml. of cytochrome + 0.4 ml. of 2×10^{-3} M riboflavin were added (Curve 2). The blank was in every respect identical with Tube 2, except that no alcohol was present during the preliminary incubation (Curve 3). Temperature, 30°; pH 8.12.

FIG. 3. Reduction of cytochrome *c* by $DPNH_2$ and riboflavin and subsequent reoxidation by cytochrome oxidase. Abscissa, time in minutes; ordinate, $CyFe^{+++}$ concentration in moles per ml. $\times 10^{-8}$. Reaction mixture, 1 ml. of 10^{-1} M phosphate, pH 7.3, 0.6 ml. of 3.65×10^{-4} M $DPNH_2$ (reduced by hydrosulfite), 0.6 ml. of 2×10^{-3} M riboflavin, 0.45 ml. of 4.43×10^{-4} M Wyeth cytochrome, and 0.85 ml. of H_2O. After 30 minutes at 30°, 0.03 ml. containing 0.03 mg. of cytochrome oxidase preparation was added at the point indicated by the arrow.

in Fig. 3 shows that after partial reduction of the cytochrome by $DPNH_2$ and riboflavin the ferrocytochrome *c* could be completely reoxidized by cytochrome oxidase.

These observations demonstrate that cytochrome *c* itself is the oxidizing agent in the system. The fact that various cytochrome preparations behave identically also witnesses against the presence of a catalytic impurity.[4] The reaction involving the three components $DPNH_2$ or $TPNH_2$, riboflavin, and ferricytochrome *c* may then be postulated as follows:

$$DPNH_2 \text{ or } TPNH_2 + \text{flavin} \rightarrow DPN \text{ or } TPN + \text{flavin } H_2 \quad (1)$$

$$\text{Flavin } H_2 + 2CyFe^{+++} \rightarrow \text{flavin} + 2CyFe^{++} + 2H^+ \quad (2)$$

The next two sections deal with the study of the two individual steps in the over-all reaction.

Reduction of Ferricytochrome c by Leucoflavin—A small amount of riboflavin (about 0.2 μM) in phosphate buffer, pH 7.3, was placed in the main compartment of a Thunberg tube, equipped with gas inlet and outlet, along with a trace of Pd black, and a solution of ferricytochrome was placed in the bulb of the tube. H_2 was then passed through the tube until the riboflavin was bleached (about 15 minutes at 30°), at which time the cytochrome was tipped in. Immediate examination with a small hand spectroscope revealed the presence of the two characteristic bands of reduced cytochrome *c*. In the corresponding control, which contained no riboflavin, the reduced cytochrome bands did not appear.[5] This seemed to offer a possibility for the quantitative study of the second part of the over-all reaction; *i.e.*, the reduction of cytochrome by leucoflavin.

For the quantitative measurement of this reaction a special tube (Fig. 4) was designed to fit the Beckman spectrophotometer, which provided strictly anaerobic conditions to prevent autoxidation of the leucoflavin, and at the same time provided a means of removing the Pd prior to addition of the leucoflavin to the cytochrome. In general the riboflavin and Pd black were placed on the sintered funnel, protected from light, and the other constituents, including cytochrome, were in the main tube. All connections were sealed with paraffin. A slow stream of H_2 was bubbled through the solution in the main tube for a few minutes, under constant shaking; then the gas inlet (*e*) was raised and a steady stream of H_2 was passed for 30 minutes *over* the solution and up through the sintered funnel. When all of the leucoflavin was reduced, the flow of gas was reversed by means of a 3-way stop-cock, and the H_2 pressure, plus a small vacuum applied at *g*, forced the reduced flavin through the sintered plate into the cytochrome solution in the main tube. The tube was then disconnected, and spectrophotometric readings were begun. Between readings the tube was kept in a constant temperature bath at 30°. In order to allow the tube to fit snugly into the Beckman cuvette carrier, small adapters were made from brass cork borers, with windows for light passage and a wedge soldered on each to prevent rotation in the cuvette carrier (Fig. 4). The top of the cell compartment was covered with black sateen cloth to prevent the entry of light during readings. Each tube was calibrated with a standard solution of reduced cytochrome *c* to determine the diameter of the cell.

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[4] The demonstration that the first of these two reactions will occur without cytochrome eliminates the possibility of a catalytic impurity in the cytochrome preparations.

[5] Although Theorell has shown that Pt black suspended directly in cytochrome will bring about a slow reduction if H_2 is bubbled through the solution (17), quantitative determinations indicated that with Pd black, particularly in our set-up, the reduction of cytochrome was negligible for at least 15 minutes.

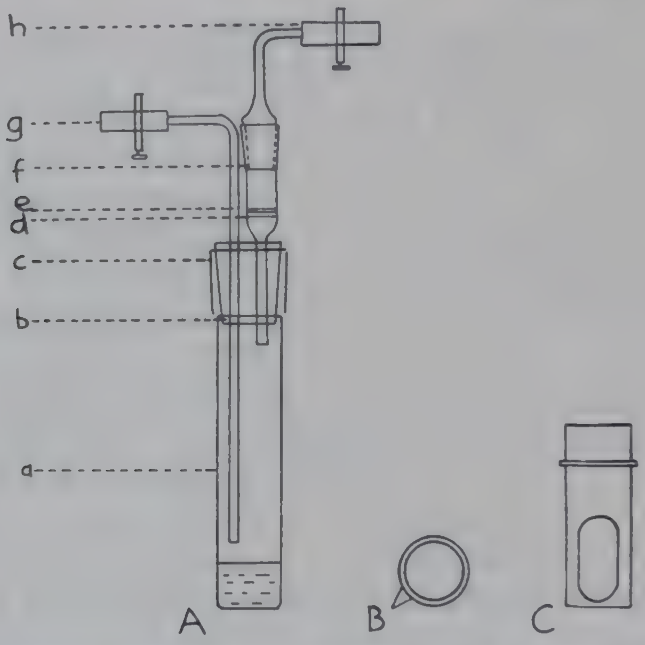

FIG. 4. Apparatus for anaerobic measurements. *A*, anaerobic tube; *B* and *C*, brass adapter, in cross-sectional and frontal view; *a*, Trubore test-tube 11 mm. inside diameter, 141 mm. long; *b*, 2-hole rubber stopper, No. 0; *c*, 14/20 outer joint; *d*, 2 ml. fine sintered glass funnel, containing Pd and the solution to be reduced; *e*, movable gas inlet; *f*, 14/35 standard taper joint; *g*, inlet rubber tubing during gassing and vacuum line outlet after gassing; *h*, outlet during gassing and inlet afterwards.

In general, the riboflavin was added in excess of the cytochrome on an equivalent basis, since losses of leucoflavin were expected in view of the imperfections of the technique. In a typical experiment, with the apparatus described above, 0.5 ml. of 2.74×10^{-4} M CyFe^{+++} (1.37×10^{-7} mole), 1.0 ml. of 10^{-1} M phosphate buffer, pH 7.3, and 1.35 ml. of H_2O were placed in the bottom part of the tube. The sintered glass funnel contained 0.15 ml. of 6.8×10^{-4} M riboflavin (1.02×10^{-7} mole), 0.5 ml. of H_2O, and about 1 mg. of Pd black. Thus the riboflavin was in 50 per cent excess over the cytochrome. Readings were begun in the Beckman apparatus within 3 minutes after mixing of leucoflavin and cytochrome. At that time only 1.53×10^{-8} mole of $CyFe^{+++}$ was left unreduced. This represents 89 per cent reduction of the added cytochrome. Readings were taken 15 minutes longer, but no additional reduction occurred. From this it can be concluded that the reduction of cytochrome by leucoflavin is a very rapid process. The incompleteness of the reduction was probably the result of some retention of leucoflavin on the sintered glass funnel or imperfectly anaerobic conditions. When 2 to 3 equivalents of leucoflavin were present, reduction proceeded to 96 to 98 per cent and, as far as we could judge, it was instantaneous. In one experiment, in which cytochrome was in good excess over leucoflavin, the reduction of $CyFe^{+++}$ was rapid at first, but slowed down toward the end of the reaction. In general, it may be stated that the reaction of leucoflavin with ferricytochrome is too fast to permit the accurate measurement of reaction rates. A corollary of this observation is the fact that in the over-all reduction of cytochrome by $DPNH_2$ the first step (oxidation of $DPNH_2$ by riboflavin) is probably the rate-limiting reaction.

In an attempt to determine the stoichiometry of the reaction, leucoflavin + $2CyFe^{+++} \rightarrow$ riboflavin + $2CyFe^{++} + 2H^+$, we modified the technique slightly by washing down the leucoflavin from the sintered glass funnel with several portions of O_2-free water. The tube containing all components was then allowed to react for 15 minutes, at which time it was opened and made to 25 ml. in a volumetric flask. An aliquot was then read in conventional Corex cuvettes. The results are summarized below.

Riboflavin added initially	2.72×10^{-7} mole
$CyFe^{+++}$ added initially	8.54×10^{-7} "
" found at end of reaction	3.99×10^{-7} "
" reduced	4.55×10^{-7} mole

$$\text{Yield} = \frac{4.55 \times 10^{-7}}{2 \times 2.72 \times 10^{-7}} \times 100 = 84\%$$

In other experiments the yield varied from 75 to 89 per cent. In view of the possible losses of leucoflavin inherent in the imperfections of the technique, this was considered a satisfactory substantiation of the reaction postulated in Equation 2.

$DPNH_2$-Riboflavin-O_2 system—In the study of the first part of the over-all reaction (Equation 1) advantage was taken of the rapid oxidation of leucoflavin by atmospheric O_2. In this system, then, riboflavin mediated electron transfer from $DPNH_2$ to O_2; the latter thus replaced ferricytochrome *c* as the oxidizing agent. Samples of $DPNH_2$, produced by enzymatic or by hydrosulfite reduction, were allowed to react with riboflavin

under aerobic conditions, and the disappearance of $DPNH_2$ was measured by the decrease in the intensity of the band at 340 mμ. The reactions were carried out in test-tubes kept in a thermostat at the desired temperature, and aliquots were momentarily transferred into quartz cuvettes when readings were taken. Since riboflavin absorbs significantly in the ultraviolet, experimental vessels were read against a blank containing all constituents, including riboflavin, except the reduced nucleotide. Preliminary measurements indicated that leucoflavin was reoxidized by the dissolved O_2 as rapidly as it was formed; therefore oxygenation made no difference in the rate of the reaction. When riboflavin was left out of the reaction mixtures, the 340 mμ band did not change for at least 30 minutes, indicating that the decrease in the intensity of this band was a reliable measure of oxidation of $DPNH_2$.

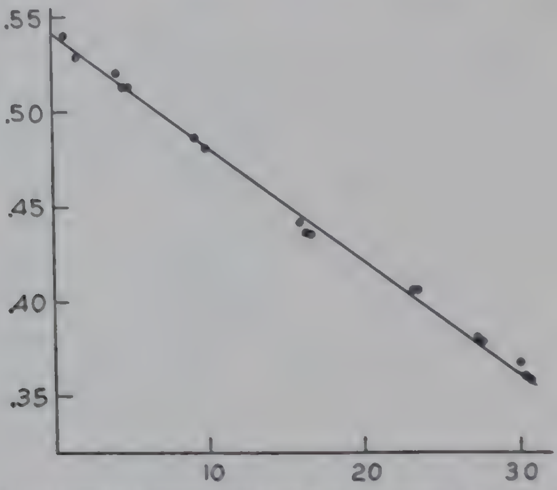

FIG. 5. Aerobic oxidation of enzymatically reduced DPN as catalyzed by riboflavin. Abscissa, time in minutes; ordinate, $1 + \log D_{340}$. The slope of the line $k = 6.0 \times 10^{-3}$. The reaction mixture contained 3.0 ml. of 10^{-1} M phosphate, pH 7.3, 0.6 ml. of 2×10^{-3} M riboflavin, 0.15 ml. of H_2O, and 1.5 ml. of enzymatically produced $DPNH_2$ (see the text). Temperature, 30°; pH 7.38. Readings taken in 1 cm. quartz cuvettes against a corresponding blank without $DPNH_2$.

The results of a very extensive series of experiments on the oxidation of $DPNH_2$ and $TPNH_2$ by riboflavin may be summarized as follows: The kinetics of the reaction are strictly first order. The velocity constant is proportional to the riboflavin concentration, the latter behaving as a true catalyst.

Fig. 5 represents a typical experiment on the oxidation of enzymatically reduced $DPNH_2$ by O_2, as catalyzed by riboflavin. A large batch of $DPNH_2$ was prepared by incubating ethanol, DPN, and alcohol oxidase in the presence of 0.1 per cent glycine and 1.4×10^{-2} M pyrophosphate, with 5×10^{-2} M dimedon at 32°. When the reduction was complete as indicated by readings at 340 mμ, *p*-chloromercuribenzoate was added to give a final concentration of 2×10^{-4} M. This stopped the action of the enzyme without interfering with subsequent use of the nucleotide preparation (18). The corresponding blank was similarly treated except that no DPN was present in the incubation mixture. Under these conditions 0.218 μM of $DPNH_2$ per ml. was formed.

An aliquot of this $DPNH_2$ solution and an aliquot of the blank were added at zero time to riboflavin in phosphate buffer at 30°, and readings were started. The results are given in Fig. 5, in which the abscissa represents the elapsed time in minutes; the ordinate is $1 + \log D_{340}$. The slope of the resulting straight line, $k = 6.0 \times 10^{-3}$, is a measure of the velocity of the reaction and depends solely on the concentration of the catalyst.[6] It can also be seen that the rate of oxidation of $DPNH_2$ is a relatively slow process, compared to the very rapid reoxidation of leucoflavin by ferricytochrome described in the previous section. Thus in the over-all reaction the reduction of riboflavin is probably the rate-limiting step. Similar first order curves were obtained by the use of hydrosulfite-reduced DPN, as well as with $TPNH_2$ and $NMNH_2$.

The catalytic function of riboflavin in this reaction is demonstrated in Fig. 6, in which the velocity constant, *k*, is plotted against the concentration of riboflavin. Thus riboflavin behaves in the same manner as cytochrome reductase in the reductase assay (3), inasmuch as the reaction rate constant is strictly proportional to the concentration of the flavin.

Reduction of Cytochrome under Anaerobic and Aerobic Conditions—In the experiments hitherto discussed the reduction of cytochrome was measured under aerobic conditions. In view of the known tendency of reduced flavins to react with O_2, it would be expected that a satisfactory stoichiometric relationship between $DPNH_2$ oxidized and $CyFe^{+++}$ reduced could be obtained only in the absence of O_2. The measurement of this relationship was one of the primary purposes of the succeeding experiment. Another purpose was to verify the prediction that the rate of the over-all reaction is limited by the first step; *i.e.*, the oxidation of $DPNH_2$ by riboflavin.

The enzymatically reduced DPN solution used in these experiments was that used in the experiment recorded in Fig. 5, which was carried out at the same time.

An aliquot of the $DPNH_2$ solution was placed in the sintered glass funnel of our anaerobic vessel (Fig. 4). All other constituents of the solution, including cytochrome, were in the main tube. A parallel experiment was set up under aerobic conditions. The pH, temperature, and concentration of the riboflavin were identical with those in the experiment on the aerobic oxidation by riboflavin and O_2 (Fig. 5). The results of these aerobic and anaerobic reactions with cytochrome are represented in Fig. 7.

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[6] We have defined *k*, the velocity constant expressed in arbitrary terms, as $-d(1 + \log D)/dt$, where *t* is the time in minutes and *D* is the optical density at 340 mμ measured in 1 cm. cells. Also $k = -d \log DPNH_2/dt$.

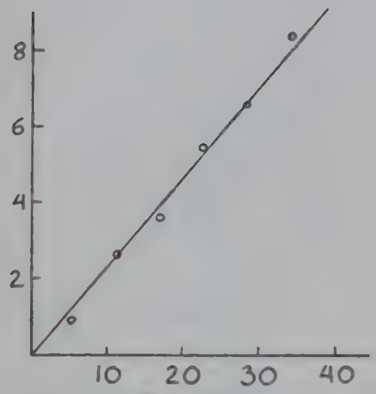

FIG. 6

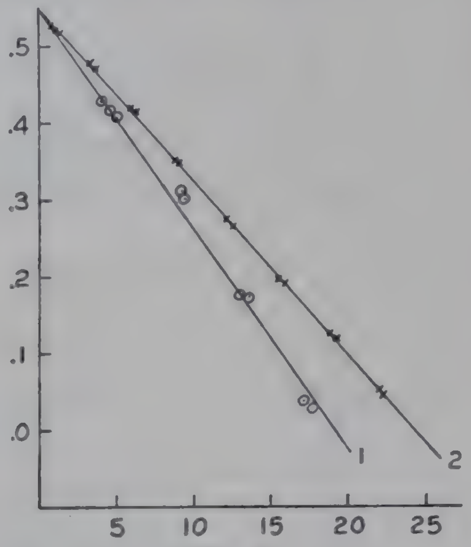

FIG. 7

FIG. 6. Relation of riboflavin concentration to the rate of oxidation of $DPNH_2$. Abscissa, moles per ml. of riboflavin × 10^{-8}; ordinate, $-d(1 + \log D)/dt \times 10^3$. Each point represents the average of at least two experiments. The reaction mixture contained 1.5 ml. of 10^{-1} M phosphate, pH 7.4 to 7.5, 0.75 ml. of riboflavin solution, 2.1 ml. of H_2O, and 0.9 ml. of 5.33×10^{-4} M $DPNH_2$, reduced by hydrosulfite immediately before the experiment. Temperature, 30°. Light absorption read in quartz cells at 340 mμ.

FIG. 7. Anaerobic and aerobic reduction of $CyFe^{+++}$ in the presence of riboflavin. Abscissa, time in minutes; ordinate, $(\log CyFe^{+++}) + 8$. Curve 1, in H_2; Curve 2, in air. In the anaerobic experiment the tube contained 1.9 ml. of 10^{-1} M phosphate, pH 7.3, 0.4 ml. of 2×10^{-3} M riboflavin, 0.2 ml. ≈ 1.26×10^{-7} mole of cytochrome *c*, and 1.0 ml. of $DPNH_2$ as in Fig. 6. The experiment was performed in the reaction vessel shown in Fig. 5, with the $DPNH_2$ held on the sintered glass funnel during the 30 minutes gassing with H_2. The reaction started upon mixing of the $DPNH_2$ with the other constituents. The aerobic tube contained 2.85 ml. of 10^{-1} M phosphate, pH 7.3, 0.6 ml. of 2×10^{-3} M riboflavin, 0.3 ml. ≈ 1.85×10^{-7} mole of cytochrome *c*, and 1.5 ml. of the same $DPNH_2$. Temperature, 30°; pH 7.4 in both experiments. Measurements at 550 mμ.

Both the aerobic and the anaerobic reduction of cytochrome *c*, like the aerobic oxidation of $DPNH_2$, followed a first order reaction.

The stoichiometry of the over-all reaction can be calculated as follows: By taking the antilogs in the ordinate of Fig. 7, the $(CyFe^{+++})$ at zero time was 3.626×10^{-8} mole per ml. At 15 minutes $(CyFe^{+++}) = 1.274 \times 10^{-8}$ mole per ml. Thus, under anaerobic conditions, *2.35×10^{-8} mole per ml.* of $CyFe^{+++}$ was reduced.

From Fig. 5, if we take the antilog corresponding to $t = 0$ and $t = 15$ minutes, the corresponding D values are 0.348 and 0.283, respectively. The difference (ΔD) = 0.065 for the 15 minute period. From the molar extinction coefficient (10), ($DPNH_2$) in moles per ml. $= D_{340}/(5.57 \times 10^6)$. Hence $0.065/5.57 \times 10^{-6} = 1.17 \times 10^{-8}$ *mole per ml.* of $DPNH_2$ oxidized. Since 1 mole of $DPNH_2 \approx 2$ moles of $CyFe^{+++}$, the corresponding equivalent in terms of cytochrome is 2.34×10^{-8} mole per ml. This is in excellent agreement with the figure obtained from anaerobic measurements of $CyFe^{+++}$ reduction at 550 mμ. This finding substantiates the fact that under anaerobic conditions in the over-all process the first step, *i.e.* the reduction of riboflavin, is the rate-limiting one, and further proves the suggested stoichiometrical relationships.

Inspection of Fig. 7 also reveals that aerobically the rate of reduction of cytochrome is only about two-thirds of the anaerobic rate.[7] The difference represents the extent of competition by dissolved oxygen; *i.e.*, the amount of leucoflavin removed via O_2. This is not a fixed ratio, of course, and the competition by O_2 will depend on the relative amounts of $CyFe^{+++}$ and O_2 present.

TABLE II

Relative Rates of Oxidation of $DPNH_2$, $TPNH_2$, *and* $NMNH_2$

Substrate	Rate of oxidation
$DPNH_2$	100
$TPNH_2$	97
$NMNH_2$	134, 138.5

Rates calculated from first order velocity constant; measurements at 340 mμ. pH 7.3; temperature, 38°. Riboflavin, 22.8×10^{-8} mole per ml.

Oxidation of Various Nucleotides by Riboflavin—In view of the relative simplicity of the ultraviolet measurements as compared with the complexity of the anaerobic technique necessary for the quantitative reduction of cytochrome, spectrophotometry at 340 mμ was used for comparison of the rates of oxidation of $DPNH_2$, $TPNH_2$, and $NMNH_2$. The rate of oxidation of $DPNH_2$, measured simultaneously with the other two nucleotides, was taken as 100 in Table II. The 78 per cent pure dried preparation of $DPNH_2$ and the $NMNH_2$ were those described under "Materials and methods." $TPNH_2$ was the product of enzymatic reduction by the *Zwischenferment* system. The latter was inactivated by maintaining it at pH 13 for 6 minutes at 25°; the resulting solution was then neutralized. This treatment left the reduced TPN unaffected.

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[7] The $-d \log CyFe^{+++}/dt$ values are 0.0302 and 0.0227, respectively.

In two experiments reduced nicotinamide mononucleotide was oxidized 34 to 38 per cent faster than $DPNH_2$, whereas in a single experiment $TPNH_2$ was oxidized at very nearly the same rate as $DPNH_2$ (Table II). All three compounds were oxidized according to a first order reaction.

Relative Efficiency of Various Flavins As Catalysts for Oxidation of $DPNH_2$—With the technique of ultraviolet spectrophotometry to measure oxidation of reduced nucleotides, the relative efficiency of various flavin derivatives as catalysts of the reaction was determined. Two minor modifications were made in the experimental method. Since only small amounts of FM and FAD were available, the concentration of flavin was lower than in previous experiments. A riboflavin control, however, at the same concentration, was run with each flavin. Secondly, when isoriboflavin was used, the time for 50 per cent disappearance of the substrate ($DPNH_2$) was calculated, since this reaction followed no definite order. It was found that the reciprocal time for 50 per cent reduction was strictly proportional to the isoriboflavin concentration; this was also true with all other flavins tested. In Table III, the catalytic activity of riboflavin is taken as 100; the rates for the other flavins are calculated from the first order velocity constant, except for isoriboflavin, as noted above.

TABLE III

Comparison of Catalytic Efficiency of Various Flavins

Catalyst	Relative rate of oxidation
Isoriboflavin	257
Riboflavin	100
" phosphate	59
Flavin-adenine dinucleotide	34
Alloxazine	0

Isoriboflavin, riboflavin, and alloxazine compared at 5.7×10^{-8} and at 11.4×10^{-8} mole per ml.; other flavins compared with riboflavin at 6.36×10^{-8} mole per ml. Source of $DPNH_2$, Ohlmeyer preparation (11); buffer, 5.7×10^{-2} M phosphate, pH 7.35; temperature, 30°; measurement at 340 mμ.

Alloxazine had no catalytic activity in the reaction. This would be expected from its relative stability to reducing agents. Among the natural compounds, the activity was greatest with riboflavin, but the analogue, isoriboflavin, had by far the greatest activity. It will be recalled that in the aerobic cytochrome reduction test, with $TPNH_2$ as substrate, isoriboflavin was also over twice as active as riboflavin. Under anaerobic conditions, with $DPNH_2$ as substrate, the rate of reduction of ferricytochrome *c* by isoriboflavin followed exactly the same type of curve as was obtained in the ultraviolet measurements, showing once more that the oxidation of the reduced pyridine nucleotide limits the rate of the over-all reaction. The relative ratio of activities of isoriboflavin and riboflavin in the anaerobic cytochrome test was 2:1, in fair agreement with the findings in Table III.

Kinetics—From the standpoint of kinetics, the simplest experimental conditions exist when the disappearance of the 340 mμ band is measured; *viz.*, the oxidation of reduced pyridine nucleotides by riboflavin and O_2. Since riboflavin acts as a true catalyst in this reaction, only the concentration of the pyridine nucleotides changes. Accordingly, the change in concentration of the latter should determine the reaction order, and, in fact, this reaction regularly yielded kinetics of the first order, regardless of the initial concentration of the pyridine nucleotides used as substrates for the oxidation. (Isoriboflavin catalysis of the reaction is an exception to this statement.) The velocity constant, however, is proportional to the flavin concentration, as expected in a catalytic reaction.

Since measurements of the rate of oxidation of leucoflavin by ferricytochrome *c* indicated that this step was very rapid, it was to be expected that the rate of the first step should determine the rate of the over-all reaction under *anaerobic* conditions. As noted above, this was the case; the reduction of cytochrome *c* measured anaerobically followed the same first order reaction as the first step.

The third and most complex case is the over-all reaction under *aerobic* conditions, for then a competition between dissolved O_2 and $CyFe^{+++}$ for the leucoflavin occurs and results in a decreased yield of ferrocytochrome. The reaction is too rapid to be compatible with a trimolecular collision and it must therefore be assumed that on reacting with 1 molecule of $CyFe^{+++}$ the leucoflavin is oxidized to the semiquinone state. This free radical could then be oxidized either by a 2nd molecule of $CyFe^{+++}$ or by O_2.

A high order of regularity for a reaction with so many variable factors could not be expected; all that could reasonably be predicted was that the rate should always be less than that under anaerobic conditions, and this was invariably true. The reaction order varied from a strict first order (as in Fig. 7) to second order (as in Table I and Fig. 1); in other instances it was not amenable to simple mathematical treatment. In the numerous instances in which a straight line was obtained when the reciprocal of the ferricytochrome concentration was plotted against time, it might possibly be inferred that the majority of the leucoflavin reacted with O_2, and the reduction of $CyFe^{+++}$ became the limiting factor, reacting with the dihydroflavin in the proportion 2:1.

Note on Stability of $DPNH_2$—Rather early in this investigation many attempts were made to study the influence of pH on the reaction by ultraviolet spectrophotometry in the DPNH$_2$-riboflavin-O$_2$ system. While it may be generally stated that between pH 9 and 5.5 the rate increases as the pH decreases, strictly quantitative measurements were hampered by the well known instability of dihydronicotinamide compounds to acidity. At and below pH 6 there was a slight but significant decrease in the D_{340} value of DPNH$_2$ controls in the course of a standard experiment.

During these studies an as yet unexplained observation was made concerning the behavior of DPNH$_2$ solutions. Using the standard conditions for measurement of DPNH$_2$ oxidation at 340 mμ, as in Fig. 5, but substituting a hydrosulfite-reduced DPNH$_2$ as substrate, we measured the velocity constant at a given pH and temperature. Several hours or a day later another aliquot was taken from the same DPNH$_2$ solution and, though the absorption of the latter at 340 mμ was unchanged, the velocity constant of its oxidation by riboflavin was in many instances increased 20 to 100 per cent. In two instances, after 2 to 3 days storage in the frozen state at pH 7.6 to 7.8, DPNH$_2$ solutions yielded a velocity constant 5 times as high as had been obtained with fresh solutions. It was first thought that the gradual formation of colloidal sulfur on standing might be responsible, but dry preparations of DPNH$_2$ (11), freed from inorganic salts, behaved qualitatively in the same way. Since fresh solutions of riboflavin and those several days old gave the same k value on the same DPNH$_2$, we felt that some transformation occurred in the nucleotide on standing which was not reflected by the D_{340} value. It might be added that TPNH$_2$, DPNH$_2$, NMNH$_2$, and even dihydronicotinamide riboside have approximately the same coefficient of extinction at 340 mμ; thus considerable variation in the molecule may be brought about which cannot be detected by spectrophotometric means. It must be emphasized that this high rate was not regularly observed with aged solutions of DPNH$_2$. In spite of much effort expended to determine the cause of this phenomenon, we are still at a loss to explain it. It can be avoided, however, by reducing the DPN immediately before use or by storing the nucleotide in the dry state (11) and using a solution of the dried material immediately after dissolving it. When the solution of the nucleotide had to be preserved for even a few hours, it was frozen by means of dry ice. In addition the true DPNH$_2$ content of preparations was ascertained by means of alcohol oxidase. Under these conditions reliable values were obtained.

DISCUSSION

On the basis of the evidence presented, it may be concluded that riboflavin, its nucleotides, and analogues are capable of catalyzing the reaction between dihydropyridine nucleotides and ferricytochrome *c*. The reason why *flavoproteins* are eminently suited for this catalytic rôle has been pointed out by Ball (2): they act as mediators between a 2 electron donor (pyridine nucleotides) and a 1 electron acceptor system (cytochrome *c*), with the intermediate formation of a relatively stable free radical of the semiquinone type (19). There is every reason to believe that free flavins act in the same manner. The reaction is thermodynamically feasible in view of the oxidation-reduction potentials: E'_0 = +0.262 volt for cytochrome *c* at neutral pH (20); approximately −0.250 volt at pH 7.8 and 30° for flavin dinucleotide and −0.232 volt for riboflavin (21); E'_0 = about −0.260 volt for pyridine nucleotides (22). Although the potential of free flavin nucleotides and of riboflavin is 120 mv. or more negative than that of the corresponding flavoproteins, and hence lies close to the range of DPN, in the presence of an oxidizing agent like O_2 or $CyFe^{+++}$ free flavins are well suited to be mediators of the oxidation.

Since ferricytochrome *c* is easily reduced by a number of agents, such as —SH compounds, H_2 + Pt, hydrazine compounds, etc., its reduction by leucoflavins is not surprising. A more striking feature of these observations is the demonstration that free flavins are able to oxidize dihydropyridine nucleotides with comparative ease. The relatively small potential span in this step might explain why this reaction should limit the rate of the over-all process.

The demonstration that free flavins can fulfil the rôle of cytochrome reductases in no way implies that this non-enzymatic process has a significant biological rôle. Although direct comparison of the turnover number of flavins with that of the flavin mononucleotide in cytochrome *c* reductase (TPN) is quite difficult, careful examination of the data presented reveals that the enzymatic process is far more efficient in terms of the flavin concentration required to reduce a given amount of cytochrome *c* per unit of time. The reasons why attachment of the flavin to the apoenzyme enhances its turnover number may now be more clearly stated. First, in such a holoenzyme combination the stability of the semiquinone radical might be increased, thus enhancing the chance of reaction with a 2nd molecule of ferricytochrome. Second, a relatively tight complex formation between reductase and cytochrome *c* (3) at the specific surface of the protein increases the probability of reaction with cytochrome and decreases loss of leucoflavin by autoxidation, an inefficient aspect of the non-enzymatic reaction. Third, formation of a relatively tight complex between the flavoenzyme and the reduced pyridine nucleotide (3) as compared with a chance collision would increase the rate of the limiting reaction; *i.e.*, reduction of the flavin by the dihydropyridine group. The same would be accomplished by raising the potential of the flavin by attachment to a specific protein (2) and thereby increasing the potential span between $DPNH_2$ and flavin, if there is indeed a direct relation between potential difference and reaction rates. Lastly, the specificity of the mediator is increased by combination with a protein. While riboflavin phosphate reacts as readily with $DPNH_2$ as with $TPNH_2$ or even $NMNH_2$, in combination with the apoenzyme from yeast it is specific for $TPNH_2$.

Naturally, the protein component required to fulfil these needs has to be a highly specific one; thus, addition of crystalline serum albumin at a concentration of 1 mg. per ml. did not significantly increase the reaction rate in our system.

The reason for the differences in the catalytic efficiencies of various flavins in our system are not clear. They do not seem to be related to the E'_0 value, inasmuch as flavin nucleotides, with a more negative potential than riboflavin, were less efficient, but isoriboflavin, with the same potential as riboflavin (23), was over twice as active.

It is hoped that these experiments provide a sound chemical basis for the function of the isoalloxazine nucleus as a mediator of electron transfer between dihydropyridine nucleotides and cytochrome *c* or molecular O_2, upon which function the most important biological rôle of riboflavin is built.

SUMMARY

1. The non-enzymatic reduction of ferricytochrome *c* by reduced pyridine nucleotides as catalyzed by various flavins has been demonstrated.

2. Of the pyridine nucleotides studied, $DPNH_2$ and $TPNH_2$ were oxidized by flavins at about the same rate; $NMNH_2$ at a faster rate. Of the flavins studied, the most effective catalyst was isoriboflavin, riboflavin, FM, and FAD being relatively less efficient in the order named. Alloxazine was unable to catalyze this reaction.

3. The reduction of cytochrome was shown to proceed in two steps; *i.e.*,

$$DPNH_2 + \text{riboflavin} \rightarrow DPN + \text{leucoflavin}$$
$$\text{Leucoflavin} + 2CyFe^{+++} \rightarrow \text{riboflavin} + 2CyFe^{++} + 2H^+$$

The first reaction proceeded as a first order reaction aerobically; the rate constant was proportional to the amount of flavin used as catalyst. The second reaction was almost instantaneous. The over-all reaction, carried out anaerobically, was limited by the rate of the first step and proceeded as a first order reaction. By determination of the amount of $DPNH_2$ oxidized in the first step and simultaneous measurement of the $CyFe^{+++}$ reduced in the anaerobic over-all reaction, it was shown that 2 $CyFe^{+++}$ molecules are reduced for 1 $DPNH_2$ oxidized. Aerobically, the over-all reaction was slower and no longer followed a first order reaction, owing probably to a competition between O_2 and $CyFe^{+++}$ for leucoflavin.

4. The probable rôle of the protein moiety of cytochrome reductases in enhancing the specificity and turnover number of the flavins is discussed on the basis of these findings.

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THE RELATIVE RATES OF METABOLISM OF NEUTRAL FAT AND PHOSPHOLIPIDES IN VARIOUS TISSUES OF THE RAT*

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Isotopic tracers have been employed to investigate numerous aspects of lipide synthesis by animal tissues, both *in vivo* and *in vitro*. In these studies attention has been centered on the formation of the carbon chains of the fatty acids, but little information has become available on the rate of entry of fatty acids into the ester linkages of various lipides. Ehrlich and Waelsch (1) compared the isotope concentration in the fatty acids of neutral fat and phosphatides in rat muscle after the administration of heavy water and found a small difference in the deuterium concentration of the two fractions. In the experiments of Barrett, Best, and Ridout (2), the feeding of deuterated linseed oil led to a somewhat higher deuterium concentration in the phospholipides than in the neutral fat of the liver. These studies, as well as those of Cavanagh and Raper (3), were designed to investigate the transport of fat in the animal body. Valuable information on the rate of synthesis and breakdown of the phospholipides in various animal tissues has been obtained with the aid of P^{32} (4, 5). However, this approach does not permit an evaluation of the relative rates of metabolism of phospholipides and other fatty acid esters.

In view of the belief that phospholipides are particularly active in fat metabolism, it seemed of interest to compare the rates at which fatty acids enter into ester linkages of different lipides. Acetic acid has been shown to serve as a precursor of the higher fatty acids both *in vivo* (6) and *in vitro* (7) and isotopic acetate can therefore be used to label the carbon chains of these tissue constituents. In the present investigation the incorporation of isotope from C^{14}-acetate into fatty acids was studied in liver slices and in the intact rat. The isotope concentrations which were found in the fatty acids of phospholipides and neutral fat were compared and taken as a measure of the turnover of the two lipide fractions.

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EXPERIMENTAL

Isotopic acetic acid was prepared from CH_3MgBr and $C^{14}O_2$.

Liver Slice Experiments—Slices were cut by hand from livers of male rats of the Sprague-Dawley strain, weighing 150 to 200 gm. 1.5 gm. aliquots from a large amount of pooled liver slices were placed in 13 ml. of Krebs' bicarbonate buffer. 5 mg. of glycerol, 11 mg. of pyruvic acid, and 5 mg. of C^{14}-sodium acetate were added to the incubation medium in each flask. The C^{14}-acetate had an activity of 75,000 c.p.m. The pH was 7.4, and the tissue was incubated for 3 hours in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 at 37°. After incubation, the lipides were isolated as described below.

Feeding Experiments—All rats used in the feeding experiments were white males of the Sprague-Dawley strain, weighing 290 to 300 gm. They were kept separately in metabolism cages and given the lipide-free diet (8) for 3 days before the administration of C^{14}-acetate was begun.

6 Hour Experiment—To each of four rats was given a single dose of 0.36 mM of C^{14}-acetate by stomach tube. The acetate had a specific activity of 760,000 c.p.m. 6 hours later the rats were killed by exsanguination under ether anesthesia and the blood was collected. From one of these rats the lipides of the liver, of the pooled internal organs (kidneys, gastrointestinal tract, testes, lung, heart, and spleen), and of the carcass (tissues remaining after removal of the head) were isolated separately. The tissues of the remaining three rats were combined and the lipides were isolated from the following organs: liver, kidney plus adrenals, the intestinal tract and mesentery.

1 and 3 Day Experiment—Two rats were kept on the lipide-free diet and received 1 mM of C^{14}-acetate per 100 gm. of rat weight per day in their food. The radioactive acetate had a specific activity of 19,000 c.p.m. After 24 and 72 hours, respectively, the rats were killed and the lipides isolated from liver, the pooled internal organs, and the carcass.

Isolation of Lipides—For isolation of the lipides the tissues were extracted several times with 1:1 ether-ethanol at room temperature. The combined extracts were concentrated *in vacuo* and the residue was taken up in ether. The ether solution was clarified by centrifugation and the phospholipides were precipitated by the addition of 4 volumes of acetone and of a small amount of $MgCl_2$ in ethanol (9). After standing in the cold for 24 hours, the precipitate was separated by centrifugation. The supernatant was concentrated and the acetone precipitation was repeated to insure complete removal of the phospholipides. The combined phospholipides were washed several times with ice-cold acetone and reprecipitated from an ether solution with acetone. The material thus obtained is designated as the phospholipide fraction, while all lipide material not precipitable by acetone from an ether solution is referred to as the neutral fat.

Blood—20 ml. of plasma were obtained from the pooled oxalated blood of the four animals in the 6 hour experiment. The plasma was mixed with 120 ml. of 1:1 ether-ethanol, the precipitate was washed with ether, and the filtrate was brought to a small volume *in vacuo*. The residue was taken up in ether and phospholipides and neutral fat were separated by the procedure described above.

Isotope analyses were carried out in all but one case on the fatty acids obtained by hydrolysis of the two lipide fractions. The lipides were digested by refluxing for 2 hours in 50 per cent ethanol containing 10 per cent KOH in a stream of nitrogen. The alkaline hydrolysate was cooled and extracted several times with petroleum ether to remove unsaponifiable material. The alkaline solution was acidified, the fatty acids were extracted by petroleum ether, and the combined extracts were washed once with 5 per cent acetic acid and several times with distilled H_2O. The solvent was evaporated and isotope analyses were carried out, either on the residual total fatty acids or in some cases individually on the saturated and unsaturated fatty acids which had been separated by the lead salt procedure. Since the amount of phospholipide obtained from the plasma was very small, the material was burned directly for isotope analysis. The isotope concentration of the fatty acids of the plasma phospholipides was calculated by assuming that the plasma phospholipide consisted of lecithin (10, 11).

The fatty acids of the neutral fat fraction are derived from all lipides which are not precipitable by acetone. They include therefore the fatty acids of the triglycerides, of the cholesterol esters, and also the free fatty acids. The amount of free fatty acids present in fresh tissues has been reported to be very small (12). Therefore in the feeding experiments the fatty acids obtained from the various neutral fat fractions were presumably derived from the triglycerides and the cholesterol esters only. On the other hand it has been reported that appreciable amounts of free fatty acids are liberated from phospholipides on autolysis of liver tissue (12). It is therefore likely that in the liver slice experiments the reaction mixture contained some free fatty acids and that these were isolated together with the neutral fat fraction. Since the fatty acids from the phospholipides had a much lower isotope concentration than those from the neutral fat fraction, it is likely that the actual differences in the isotope concentration of the two fractions were greater than the analyses indicate.

From the content of cholesterol esters in various tissues it can be estimated that fatty acids linked to cholesterol may comprise as much as 25 per cent of the fatty acids of the neutral fat. While it is likely that the fatty acids of neutral fat in various organs are mainly derived from triglycerides, cholesterol esters and free fatty acids constitute a relatively large portion of the neutral fat fraction (13) in plasma, in which, therefore, the isotope concentration of the fatty acids of the triglycerides may be quite different from that of the fatty acid mixture analyzed.

Analysis for Essential Fatty Acids in Rat Liver—Tissue fat contains polyethenoid fatty acids which are of exogenous origin. These fatty acids, which are part of the unsaturated fatty acid fraction, would not be expected to contain any isotope under the present experimental conditions (14). In order to determine the isotope concentration of the monounsaturated fatty acids it is necessary to know the content of polyunsaturated fatty acids in liver fat. From 60 gm. of rat liver the phospholipides and the neutral fat were separated as described above. The contents of arachidonic, linoleic, and linolenic acids were determined according to the spectrophotometric method of Mitchell, Kraybill, and Zscheile (15). The oleic acid content was determined from the iodine number, and the per cent of saturated fatty acids was calculated by difference[1] (the results obtained in two such experiments are given in Table I).

TABLE I

Fatty Acid Composition of Phospholipide and Neutral Fat of Normal Rat Liver

	Per cent total fatty acids			
	Phospholipides		Neutral fat	
	(A)	(B)	(A)	(B)
Arachidonic acid	23.2	23.5	11.5	10.8
Linolenic acid	0.0	0.0	0.16	0.36
Linoleic "	7.6	7.6	15.9	16.1
Oleic acid	39.0	39.1	62.8	64.4
Saturated fatty acids	30.1	30.9	9.6	8.3
Iodine No.	126.4		124.2	

Isotope Analyses—All compounds were burned in a micro combustion apparatus and the CO_2 was precipitated as $BaCO_3$. The $BaCO_3$ was suspended in methanol and deposited on cups of 3.47 sq. cm. area. The samples were counted with a gas flow counter and corrected for thickness. All C^{14} concentrations are reported in counts per minute.

DISCUSSION

The lipides of animal tissues are mixtures of several components and cannot be separated readily into well defined fractions. The two lipide fractions which have been compared in the present study have been distinguished on the basis of their solubility in acetone. This procedure is commonly employed to separate phospholipides from neutral fat, but it should be realized that each of the two fractions is a mixture of several components. Nevertheless, a comparison of the isotope concentrations in the two fractions should give an indication of the relative metabolic activity of the phosphorus-containing lipides and the so called neutral fat.

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[1] These analyses were kindly performed by Dr. L. R. Dugan and Miss M. Petheram in the Research Laboratories of the American Meat Institute Foundation.

Liver Lipides—When rat liver slices were incubated with C^{14}-acetate as a fatty acid precursor, the fatty acids obtained from neutral fat were found to have an isotope concentration about twice as high as that of the fatty acids from the phospholipides (Table II). Since in rat liver slices acetate carbon is incorporated at a much faster rate into the saturated than into the unsaturated fatty acids (16), differences in fatty acid composition could be responsible in part for these results. However, separate analyses demonstrated that both saturated and unsaturated fatty acids of the neutral fat had a much higher isotope concentration than those of the phospholipides. The data for the unsaturated acids reflect the relative rates of incorporation of the monounsaturated fatty acids only because the polyunsaturated acids do not contain any isotope under the present conditions (14). The actual isotope concentrations of the monounsaturated fatty acids (Column B, Table II) were calculated by subtracting the content of these unlabeled fatty acids.

TABLE II

Incorporation of C^{14}-Acetate into Fatty Acids of Neutral Fat and Phospholipides in Rat Liver Slices

Experiment No.		C^{14} concentration, c.p.m.			
		Total fatty acids	Saturated fatty acids	Unsaturated fatty acids	
				Total (A)	Monounsaturated* (B)
1	Neutral fat	132	325	41	59
	Phospholipides	80	127	15	27
2	Neutral fat	64	99	29	42
	Phospholipides	28	50	12	22

For details, see "Experimental" in the text.

* Calculated from the data in Table I.

In order to investigate whether differences in metabolic rates of various lipides could be detected also in the intact animal, C^{14}-acetic acid was administered to adult rats for various periods of time. 6 hours after a single administration of labeled acetate by stomach tube the isotope concentration in the total, the saturated, and the unsaturated fatty acids of neutral fat of liver was found to be significantly higher than in the corresponding fractions of the liver phospholipides (Table III). Therefore, *in vivo* as well as *in vitro*, the newly synthesized fatty acids are more rapidly incorporated into the ester linkages of neutral fat than of liver phospholipides and it would appear that phospholipides cannot be obligatory intermediates in the synthesis of neutral fat.[2] Zilversmit, Chaikoff, and Entenman (17) have reached the same conclusion with respect to the rôle of phospholipides in the intestinal absorption of fat. The present findings are also in accord with the results obtained by Cavanagh and Raper (3). These investigators fed deuterated linseed oil to rats and determined the deuterium concentration in the phospholipides and neutral fat of various organs after successive time intervals. Their finding that between the 10th and 24th hours following the administration of labeled fat the deuterium concentration in the neutral fat of liver decreased much faster than that of the phospholipides indicates that the replacement of the fatty acid was more rapid in neutral fat.

TABLE III

Isotope Concentration in Fatty Acids of Neutral Fat and Phospholipides of Liver 6 Hours after Feeding of C^{14}-Labeled Acetate

Rat No.		C^{14} concentration, c.p.m.			
		Total fatty acids	Saturated fatty acids	Unsaturated fatty acids	
				Total (A)	Monounsaturated* (B)
1	Neutral fat	2600	3100	2500	3600
	Phospholipide	1500	1900	1200	2100
2, 3, 4	Neutral fat	2500	2600	2500	3600
Pooled	Phospholipide	1700	2200	1300	2300

* Calculated from the data in Table I.

In the experiments lasting 1 and 3 days (Table IV), in which the animals received the labeled acetate admixed with the diet, the isotope concentrations of the fatty acids of the neutral fat and phospholipides of liver were essentially the same. The fact that differences can no longer be observed after these time intervals suggests that the regeneration of the ester bonds is a very fast process in both lipides.

Plasma Lipides—The isotope concentrations of the lipides of plasma in the 6 hour experiment were found to be very high and to be exceeded only by those of the lipides in liver (Table V). Here also the isotope concentration of the fatty acids of the neutral fat fraction is higher than that of the phospholipide. Since only total fatty acids were analyzed, it is important to consider the fatty acid composition of the plasma lipides. An exact fatty acid analysis of these lipides is available only for ox plasma (13). In contrast to the neutral fat from various tissues, that of plasma contains only a small percentage of triglycerides and a large amount of free fatty acids and cholesterol ester. The fatty acids linked to cholesterol are known to consist to a large extent of essential fatty acids. It is therefore likely that the isotope concentration in the saturated and monounsaturated fatty acids of the neutral esters in plasma is appreciably higher than the analytical data indicate.

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[2] This conclusion will not be valid if one of the components of the phospholipide fraction has a very much higher rate of turnover than is indicated by the C^{14} concentration in the total mixture. However, no evidence exists at present to support the latter assumption.

TABLE IV

Isotope Concentration in Total Fatty Acids of Neutral Fat and Phospholipides of Rat Tissues after Feeding of C^{14}-Acetate

Rat No.	Duration	C^{14} concentration, c.p.m.					
		Liver		Pooled internal organs		Carcass	
		Neutral fat	Phospholipide	Neutral fat	Phospholipide	Neutral fat	Phospholipide
	hrs.						
5	24	230	260	29	115	23	32
6	72	340	360	42	155	31	76

TABLE V

Isotope Concentration in Total Fatty Acids of Neutral Fat and Phospholipides 6 Hours after Feeding of C^{14}-Labeled Acetate

	C^{14} concentration, c.p.m.	
	Neutral fat	Phospholipides
Pooled internal organs	77	169
Kidney plus adrenals	68	344
Intestinal tract	118	430
Mesenterial fat	29	75
Carcass	81	87
Plasma	1600	1400

In view of the fact that the phospholipides comprise a relatively small fraction of the total plasma lipides (13) and also have a relatively low isotope concentration, it is unlikely that the phospholipides are the major vehicle for fat transport.

The isotope concentration of the fatty acids of the plasma phospholipides was approximately 90 per cent that of the fatty acids in the liver phospholipides. Since liver is the only organ in which the isotope concentration of the phospholipides is greater, the plasma phospholipides must have originated from this organ. The present data are in agreement with previous experiments with P^{32} (5, 18, 19), which demonstrated that the liver is the site of synthesis for plasma phospholipides. A direct comparison of the radioactivity in the neutral fat fraction of plasma and liver has little meaning, as their composition is widely different.

Internal Organs—In the 6 hour experiment the relation between the isotope concentrations of phospholipides and neutral fat in the combined internal organs and in those internal organs which were separately analyzed was found to be the reverse of that existing in the liver (Table V). At all time intervals the radioactivity of the fatty acids from phospholipides exceeded that of the fatty acids from neutral fat several fold (Table IV). It is of interest that this is true not only for the parenchymatous lipides of the kidney and adrenals, but also for mesenterial fat, which is a representative depot fat. While it is evident that in these internal organs the rate of turnover of phospholipide is greater than that of neutral fat, the present data do not indicate to what extent the lipides in these organs are replaced by interchange with the lipides of the blood and by synthesis *in situ*.

The level of radioactivity in the neutral fat from all extrahepatic tissues, including carcass, was about the same. On the other hand, the metabolic activity of the phospholipides differed widely from organ to organ and was lowest in carcass and mesenterial fat. While in the extrahepatic tissues discussed above the isotope concentration in the phospholipides was several times greater than that of the neutral fat, this was not the case for the lipides from carcass. In one of the three experiments (Table IV) the isotope concentration of the phospholipides was significantly higher than in the neutral fat, but the data for carcass as a whole indicate relatively small differences in metabolic activity between the two fractions.

The present findings do not permit a generalized statement concerning the relative metabolic activity of phospholipides and neutral fat in the animal organism. Thus the results obtained for liver indicate that ester bond formation is a faster process for triglycerides than for phospholipides. In this organ the observed radioactivities reflect primarily the synthesis of fat. On the other hand, the appearance of labeled lipides in extrahepatic organs must to a large extent result from the uptake of lipides from the blood stream. Irrespective of the mechanism by which replacement occurs in these organs, these processes are decidedly faster for phospholipides than for neutral fat.

SUMMARY

1. The incorporation of isotopic carbon from C^{14}-acetate into the fatty acids of neutral fat and of phospholipides has been studied in isolated liver and in the intact rat.

2. The results obtained show that in liver the rate of regeneration of neutral fat is faster than that of phospholipides. It is concluded that the phospholipides are not obligatory intermediates in the synthesis of triglycerides.

3. In all other internal organs, including mesenterial fat, the isotope concentrations found in the phospholipides were markedly higher than in neutral fat.

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THE RATES OF SYNTHESIS OF FATTY ACIDS AND CHOLESTEROL IN THE ADULT RAT STUDIED WITH THE AID OF LABELED ACETIC ACID*

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The rates of synthesis and destruction of organic tissue constituents in the animal organism were first investigated by Schoenheimer and Rittenberg (1, 2). By administering heavy water to mice they were able to measure the rates of regeneration of fatty acids and cholesterol. Several similar studies have been carried out with mice (3) and rats (4, 5), and approximate values for the turnover of the lipides in liver, carcass, and the total animal body have been reported.

The rate of turnover of a body constituent is not necessarily a constant, but may vary with the animal species, age, the composition of the diet, etc. In several of the previous turn-over studies, fat has not been rigidly excluded from the diet, while in others net formation of tissue constituents took place. In the earlier experiments deuterium has been the tracer throughout, on the assumption that the rate of incorporation of deuterium from the heavy water medium is a true measure of the rate of synthesis of the compound in question. Since no such assumption need be made when a carbon isotope is used in the tracer molecule, the rate of turnover of fatty acids and cholesterol has been reinvestigated with the aid of acetic acid labeled by C^{14} in the carboxyl group. The present study had the additional object of determining the extent of utilization of acetic acid in the synthesis of fatty acids and cholesterol.

Adult rats were used as the experimental animals. They were maintained at constant body weight on a diet completely devoid of fat and cholesterol. γ-Phenyl-DL-α-aminobutyric acid was added to the diet, and its acetyl derivative isolated from the urines.

EXPERIMENTAL

Isotopic potassium acetate labeled in the carboxyl group with C^{14} was prepared from CH_3MgBr and $C^{14}O_2$.

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Feeding Experiments—Eight male rats of the Sprague-Dawley strain, weighing 290 to 300 gm. each, were kept separately in metabolism cages and given 12 to 15 gm. per day per rat of the following diet: vitamin-free casein 20, sucrose 65, non-nutritive material[1] 10, and standard salt mixture[2] 5.

To each kilo of the above diet, vitamins were added in the following amounts: thiamine 4 mg., riboflavin 10 mg., calcium pantothenate 16 mg., inositol 1 gm., *p*-aminobenzoic acid 30 mg., choline 1 gm., folic acid 2 mg., biotin 0.1 mg., β-carotene 5.4 mg., calciferol 0.03 mg., 2-Me-1-4-naphthoquinone 2 mg., α-tocopherol 60 mg., and pyridoxine 4 mg.

The requirement for essential fatty acids was met by administering 15 mg. of methyl linoleate per rat per day. Methyl linoleate and the fat-soluble vitamins, dissolved in tripropionate, were added to the ration each day.

Isotopic acetic acid, having a specific activity of approximately 1 μc. per mM, was thoroughly mixed with the ration and fed at a level of 1 mM per 100 gm. of rat tissue per day. A sample of the acetic acid was converted to silver acetate. It showed an activity of 19,000 c.p.m., counted as barium carbonate.

Pairs of rats were sacrificed after the 1st, 3rd, 10th, and 30th days (Experiment A). Fatty acids from liver and eviscerated carcass were isolated according to customary methods and separated into saturated and unsaturated fractions by the lead salt procedure. Cholesterol from liver was isolated as the digitonide. Carcass cholesterol was obtained from the unsaponifiable fraction by repeated recrystallizations.

The urines were collected and *N*-acetylphenylaminobutyric acid was isolated and purified according to the method described previously (6). In the 1, 3, and 10 day experiments the urine excreted by each individual rat during the entire experimental period was pooled for isolation of acetylphenylaminobutyric acid. The isotope concentrations found are, therefore, average values for the respective periods. The quantities of phenylaminobutyric acid added to the diet were 150, 50, 25, and 25 mg. per day, respectively, in the 1, 3, 10, and 30 day experiments. In the 30 day experiment acetylphenylaminobutyric acid was isolated separately from the urine excreted during the first 17 day period and from that excreted during the last 13 days. In another 30 day experiment (Experiment B), two rats received 50 mg. of DL-phenylaminobutyric acid per rat per day. The urines from eight successive periods were collected and analyzed separately. The isotope concentrations of the acetyl groups of acetylphenylaminobutyric acid in Experiment A are given in Table I and those for Experiment B, in Fig. 4.

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[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).

Isotope Analysis—All compounds were burned in a micro combustion apparatus and the CO_2 was precipitated as $BaCO_3$. The $BaCO_3$ was suspended in methanol and deposited on cups of 3.5 sq. cm. area. The samples were counted with a flow gas counter and corrected for thickness. All values are reported in counts per minute. The probable error of the analyses is ±5 per cent. The C^{14} concentrations in cholesterol samples which had been isolated as the digitonides were obtained by multiplying the analytical values by 3.07. The C^{14} concentrations of the acetyl groups in acetylphenylaminobutyric acid were calculated by multiplying the found values by 6.

TABLE I

Isotope Concentration of Acetyl Groups of Acetylphenylaminobutyric Acid: Experiment A

	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7		Rat 8	
Period of urine collection, *days*	1	1	3	3	10	10	1–17	18–30	1–17	18–30
Acetyl groups, *c.p.m.*	1540	1460	1650	1550	1900	1760	2000	2050	2150	1900

DISCUSSION

The customary method of calculating the half time[3] of a tissue constituent from the rise of its isotope concentration with time is valid only if the isotope concentration of the precursor stays constant throughout the experimental period. It has previously been assumed that after the feeding of labeled acetate the isotope concentration of the acetic acid which is available for endogenous synthesis at any given time is indicated by the isotope concentration of *N*-acetyl groups which are excreted after the administration of foreign amines (7). The isotope concentration of such acetyl groups does not change with time over feeding periods lasting as long as 3 months, if deuterioacetate is the test substance (8). In the present experiments with acetic acid labeled by a carbon isotope the same procedure has been used. Phenyl-DL-aminobutyric acid was fed simultaneously with the labeled acetate and consecutive samples of the excreted acetyl derivatives were analyzed. As shown in the data of Table I, the isotope concentration of the acetyl groups, contrary to the findings with deuterioacetate, changes with time when carbon-labeled acetic acid is the precursor and increases by approximately 30 per cent in the course of 30 days. It must be assumed that this rise reflects an increase of the isotope concentration of the acetic acid which serves as the precursor for fatty acids and cholesterol. In order to determine the half time it is therefore necessary to recalculate the found isotope values for fatty acids and cholesterol on the basis of the changing isotope concentration of the acetic acid in the metabolic "pool." These values have therefore been adjusted by calculating the isotope concentrations of the tissue constituent in per cent of the average isotope concentration in the acetyl groups excreted during the corresponding time interval.

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[3] The time at which the isotope concentration of a body constituent is one-half of the value which would be reached at infinite time.

Fatty Acids—In Fig. 1 are plotted the isotope concentrations in the saturated and unsaturated fatty acids of liver after various time intervals, expressed in per cent of the isotope concentration of the acetyl groups. The saturated fatty acids reach half of their maximal isotope concentrations in less than 1 day, and the unsaturated acids in about 2 days. These values are in good agreement with the half life time of 1.9 days obtained with D_2O by Stetten and Boxer (5) for the total fatty acids of rat liver.

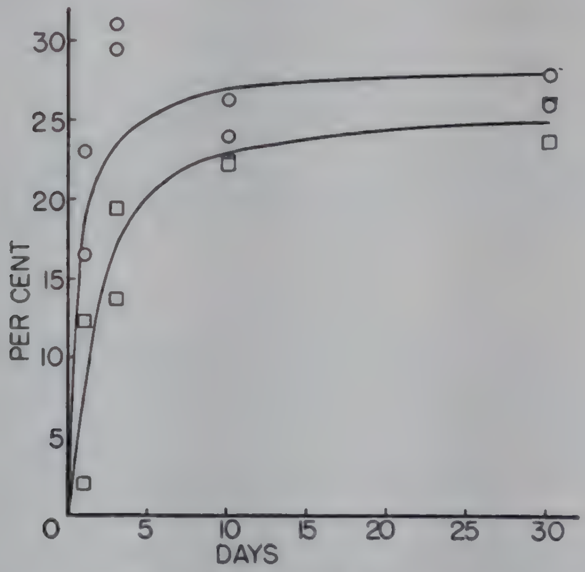

Fig. 1. Isotope concentrations of saturated (○) and unsaturated (□) fatty acids of liver, expressed in per cent of the isotope concentrations of the acetyl groups excreted at corresponding time intervals.

The estimation of the half time of the liver fatty acids may be subject to considerable error because the isotope concentrations rise very sharply during the initial phase of the experiment. When the half time of a compound is as short as 1 day, variations in food intake, etc., during the experimental period may have an appreciable effect on the isotope concentration of the labeled product. A more accurate value of the half time is therefore not obtainable from feeding experiments with the analytical technique which is used at present.

The isotope concentration of the carcass fatty acids did not reach a maximum within the experimental period of 30 days and consequently the half time could not be determined directly from the data, as was possible for the liver fatty acids. In the experiments with deuterioacetate of Ponticorvo, Rittenberg, and Bloch (8) the isotope concentration of the carcass fatty acids reached the same final value as the liver fatty acids when the feeding period was extended beyond 1 month. We have therefore assumed that in the present experiment also the isotope concentration in the carcass fatty acids will ultimately attain the same maximal value as the fatty acids of the liver. Furthermore, by plotting the data semilogarithmically with the first order rate equation,[4] $\ln I/(I - i) = kt$, a straight line is obtained only when the values used for I are those of the saturated and unsaturated fatty acids in the liver. This supports the assumption that the isotope concentrations in the fatty acids in carcass will ultimately be the same as in the fatty acids of liver. After plotting of the data according to the above equation (Fig. 2), k can be estimated from the slope of the curve and the half time can be obtained from the relation $t_{\frac{1}{2}} = (\ln 2)/k$. Values of 16 to 17 days for the saturated and 19 to 20 days for the unsaturated fatty acids of carcass are found.

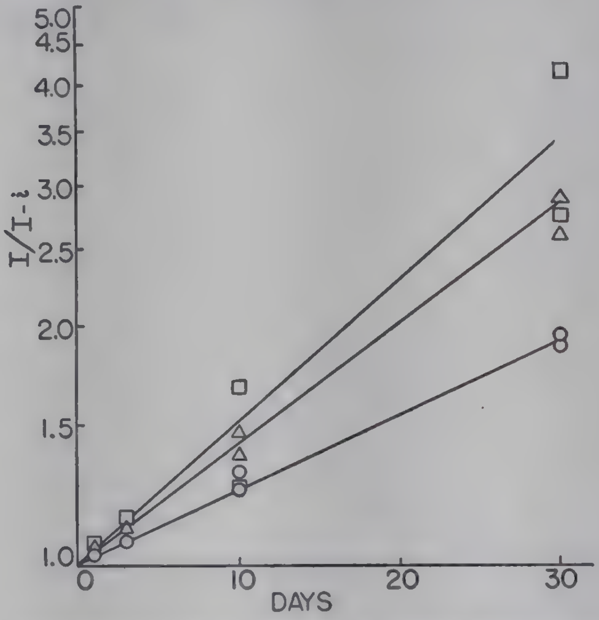

FIG. 2. Isotope concentrations of saturated (□) and unsaturated (△) fatty acids and cholesterol (○) of the carcass, plotted as the function $\ln I/(I - i) = kt$. I and i are expressed in per cent of the isotope concentrations of the acetyl groups excreted at corresponding time intervals.

The values obtained in the present investigation are appreciably higher than the half life time of 9 days found by Bernhard and Bullet (4) in experiments on rats with heavy water. This difference is unexpected because the half time should be independent of the nature of the tracer molecule used. A variation in the behavior of different rat strains is the only apparent explanation for the differences observed in the two laboratories.

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[4] I, isotope concentrations at infinite time and i, isotope concentration at time t.

It may be noted that the unsaturated fatty acids are synthesized at a slightly slower rate than the saturated fatty acids while their maximal isotope concentrations are not appreciably lower. As the unsaturated fatty acid fractions include the essential polyethenoid fatty acids which presumably are unlabeled (9), the isotope concentrations of the monounsaturated acids must be at least as high as those of the saturated fatty acids (10). On the other hand, in investigations with heavy water as a tracer, the maximal isotope concentration of the unsaturated fatty acids was found to be not more than half that in the saturated acids from the same organ (2–4). It should be pointed out that in some of the earli investigations the diets employed were not rigorously freed of fat. Th may account in part for the observed differences. The fact that in th present experiments the saturated and the monounsaturated fatty aci show the same isotope concentration at infinite time suggests that acet acid is used to the same extent in their synthesis. On the other hand, t differences in the maximal deuterium concentration observed after admi istration of heavy water probably reflect a difference in the mechanis of synthesis for saturated and unsaturated fatty acids as suggested Bernhard and Bullet (4) and by findings from this laboratory (11, 1

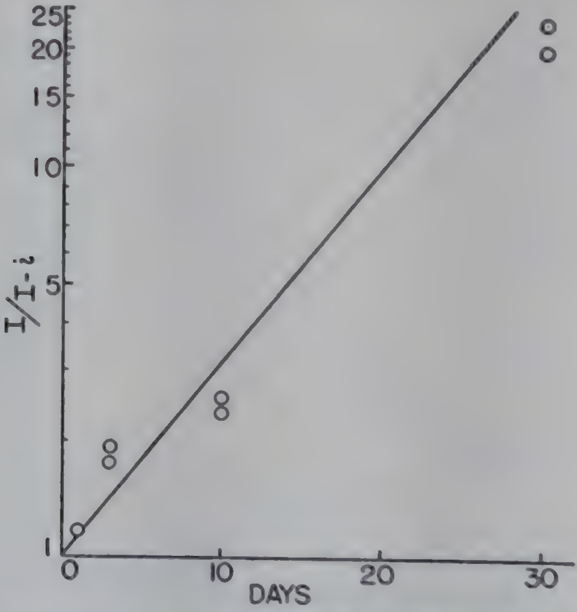

Fig. 3. Isotope concentration of liver cholesterol plotted as the function ln I $(I - i) = kt$. I and i were calculated in the same manner as explained in Fig. 2.

Cholesterol—The data for cholesterol have been plotted semilogarit mically as in the case of the fatty acids (Fig. 3). For liver cholestero alf time of about 6 days is obtained. Since the isotope concentration n the cholesterol of carcass (Fig. 2) rises slowly and, like that of the arcass fatty acids, does not reach its maximum within the experimental eriod, the half time has been calculated under the assumption that its sotope concentration reaches the same maximal value as the liver cho-esterol. On this basis the half time in carcass is calculated to be 31 to 32 days. Rittenberg and Schoenheimer (2) estimated the half life time of total body cholesterol in mice to be 15 to 25 days.

Utilization of Acetate for Lipide Synthesis—In previous studies (7, 11, 8) the extent of utilization of acetate for the synthesis of a body constituent has been estimated by referring the isotope concentration of the product in question to the isotope concentration of excreted *N*-acetyl groups. The assumption has been made that the isotope concentration of these acetyl groups is the same as that of the acetate at the site of synthesis.

In the present experiment the maximal isotope concentration in the liver cholesterol amounts to 26 per cent of that in the acetyl groups. If the 2-carbon atoms of acetic acid were utilized equally in cholesterol synthesis, 26 per cent of all carbon atoms would have their origin in acetic acid. Actually in experiments on cholesterol formation in rat liver slices it has been found (13) that the methyl and carboxyl carbons in acetic acid enter cholesterol in a ratio of 1.27:1. It seems likely that *in vivo* also a larger proportion of the sterol carbon atoms is supplied by the methyl group of acetic acid. Therefore the value of 26 per cent represents the minimal utilization of the acetic acid molecule. This value is substantially lower than previous estimates (7, 14, 8) on acetate utilization for steroid synthesis.

Recently the isotope concentration of some individual carbon atoms of cholesterol, which had been synthesized in liver slices in the presence of carbon-labeled acetate, has been determined (13). The results obtained indicated that every individual carbon atom of cholesterol can be derived from acetic acid. Since no other specific carbon source of cholesterol has been found so far, and since in the present experiment the cholesterol contained only one-fourth of the isotope concentration of the excreted acetyl groups, it would appear that the 2-carbon fragments which serve as the immediate precursor of cholesterol and of the acetyl groups excreted in the urine, respectively, do not have the same isotope concentration. No decision can be made at the present time as to whether there exist other sources of the immediate sterol precursor or whether it is erroneous to assume that the isotope concentration in the excreted acetyl groups reflects the true isotope concentration of acetic acid in the metabolic "pool."

Similar considerations apply to the rôle of acetic acid as a carbon source for fatty acids. The finding in the present experiments that the maximal isotope concentration in the liver fatty acids was about 25 per cent that of the acetyl groups indicates that at least one-fourth of the carbon atoms of the fatty acids is furnished by acetic acid. The present value of acetate utilization for fat synthesis is in good agreement with previous estimates from experiments on mice with carbon-labeled acetic acid (14) and on rats with deuterioacetic acid (8).

In the case of the fatty acids also, evidence has been obtained that acetic acid can furnish every individual carbon atom of the molecule (14). Again, the values of acetate utilization, calculated from the present and from earlier experiments, can be minimal values only for the reasons mentioned above in the discussion of cholesterol synthesis. The two processes differ, however, in that 2-carbon units derived from pyruvic acid are a source of carbon atoms for the higher fatty acids but do not contribute proportionally to the acetylation of foreign amines (11). The isotope concentration of the *N*-acetyl groups after feeding labeled acetate is therefore a measure of the acetate pool in a restricted sense only.

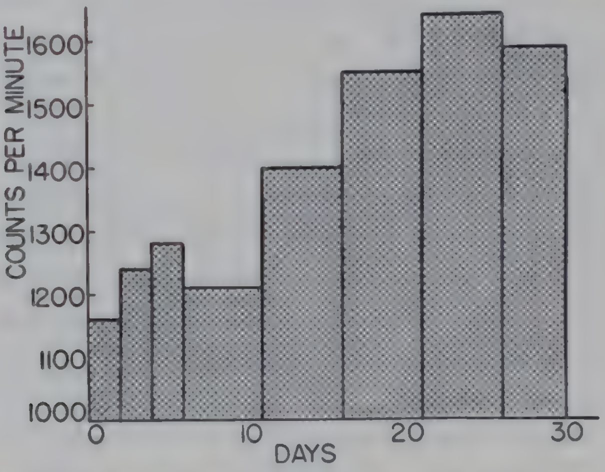

FIG. 4. Experiment B: isotope concentrations of excreted acetyl groups

The observation that the isotope concentration of the excreted acetyl groups rises gradually on continued feeding of labeled acetate was confirmed by a second long term experiment in which the isotope concentration of the acetyl groups was determined in smaller time intervals. The results are given in Fig. 4. The increase of the isotope concentration above the initial value in the course of the 30 day period was again about 30 per cent. Since the amount of exogenous labeled acetate was constant, the observed rise must be caused by labeled tissue constituents, which in turn are metabolized to yield labeled acetyl groups. Compounds which are known to be synthesized from acetate as a precursor are fatty acids (14), cholesterol (15), heme (7), and purines (16). On the other hand the formation of acetic acid as a catabolic product has been demonstrated only for fatty acids (6) and for one of the ketogenic amino acids (17). Under the present experimental conditions the higher fatty acids are the only compounds which can contribute significant quantities of labeled acetyl groups to the acetic acid pool. The question arises why the isotope concentration in the excreted acetyl groups increases with time when carbon-labeled acetate is the test substance but stays constant when deuterioacetate is used (8). It is likely that during the breakdown as well as during the synthesis of deuterio fatty acids the major part of the deuterium will be replaced by ordinary hydrogen from the body fluids. The deuterium concentration in a 2-carbon split-product arising during the breakdown of a deuterio fatty acid will thus be too small to affect the isotope concentration of the excreted acetyl groups. On the other hand, such a loss of isotope cannot occur with fatty acids which contain isotopic carbon.

From the data furnished by the present experiment, the quantities of acetyl groups formed by the breakdown of fatty acids can be estimated. In this calculation only the carcass fatty acids will be considered. Under the assumption that a 300 gm. rat has a fatty acid content of 20 gm., with an average half time of 18 days, it is calculated that a total of about 22 mM of acetic acid can be produced from this source per day. This acetate will have the same isotope concentration as the fatty acids from which it is formed. After feeding 3 mM of acetate per rat per day with an isotope concentration of 19,000 c.p.m., the acetyl groups in the 1 day experiment were found to contain about 1500 c.p.m. (Table I). The 3 mM of dietary acetate had therefore been diluted by about 35 mM of unlabeled endogenous acetate. On the 30th day of the experiment, the 3 mM of dietary acetate will mix with the same quantity of endogenous acetate which now, however, has an isotope concentration at least as high as that of the carcass fatty acids; *i.e.* 360 c.p.m. This contribution will raise the isotope concentration in the mixture of exogenous and endogenous acetate to be employed for the acetylation reaction from 1500 to 1800 c.p.m. The actual isotope concentration in the acetyl groups excreted between the 17th and 30th days was found to have an average value of 2000 c.p.m. Therefore the degradation of the fatty acids in the carcass alone can account for about 60 per cent of the observed rise. The contribution of labeled acetyl groups from fat in other tissues must also be considerable but is difficult to evaluate because the rate of turnover of the fatty acids in the internal organs has not been determined. Moreover, in the case of the liver the observed rate of turnover probably reflects not only the synthesis and breakdown of the fatty acids, but also the transport of fatty acids to and from extrahepatic tissues. In any case the results are consistent with the assumption that the increasing isotope concentration in the excreted acetyl groups is due to the formation of labeled 2-carbon units from the higher fatty acids. This is in accord with the view that the size of the acetyl pool as indicated by the acetylation reaction can be accounted for by the known metabolic activity of tissue fat (7). Other sources such as carbohydrate may give rise to intermediates which behave like 2-carbon fragments in some respects, *e.g.* in the synthesis of fatty acids, but which do not contribute proportionally to the acetic acid pool as measured by the acetylation reaction.

SUMMARY

1. The rate of synthesis of fatty acids and cholesterol in the adult rat has been determined with the aid of acetic acid labeled by C^{14}. The half times for the saturated and unsaturated fatty acids were found to be about 1 day and 2 days, respectively, in liver and 16 to 17 days and 19 to 20 days, respectively, in the carcass. The half time of cholesterol was found to be 6 days in liver and 31 to 32 days in carcass.

2. Minimal values have been calculated for the utilization of acetic acid in the synthesis of cholesterol and of fatty acids in the adult rat.

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THE OCCURRENCE OF γ-AMINOBUTYRIC ACID IN YEAST EXTRACT; ITS ISOLATION AND IDENTIFICATION

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Partition chromatography on paper has proved an invaluable tool in separating the amino acids in protein hydrolysates and biological extracts (1–5). The amino acids migrate to characteristic positions on paper chromatograms, as revealed by a color reaction with ninhydrin. During the course of studies with yeast extract (Difco), this material was subjected to mild acid hydrolysis, and the hydrolysate was examined chromatographically. The presence in appreciable quantity of an amino acid of unknown nature was revealed. This amino acid was isolated from the acid-treated yeast extract and identified as γ-aminobutyric acid. Further experiments demonstrated that it is present in the free state in untreated yeast extract.

The isolation was effected by means of partition chromatography on a starch column (6), by use of *n*-butanol-acetic acid-water as the solvent system. Separation of the amino acids by the column was traced by paper partition chromatography of samples of the effluent.

EXPERIMENTAL

50 gm. of Bacto yeast extract[1] were dissolved in 500 ml. of 6 N hydrochloric acid and the solution was autoclaved for 1 hour at 120°. The cooled digest was treated with Darco G-60 and freed of chloride ions by successive treatments with lead oxide and silver carbonate. The filtrate from these operations was saturated with hydrogen sulfide, decolorized with Darco G-60, and concentrated to a volume of approximately 150 ml. This solution contained 113 mg. of solids per ml.

Partition Chromatography on Paper[2]—A two-dimensional paper chromatogram of the chloride-free acid digest of yeast extract (Fig. 1) was prepared and developed according to the "ascending" method of Williams and Kirby (7). A comparison of this chromatogram with one obtained by using a mixture of the known naturally occurring amino acids revealed that an amino acid of unknown nature was present in the acid digest of yeast extract. In a similar manner the "unknown" amino acid was shown not to be identical with a number of synthetic amino acids.

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[1] Obtained from the Difco Laboratories, Detroit, Michigan.

[2] Whatman No. 1 filter paper was used throughout this work.

Several different solvent systems were tested in conjunction with one-dimensional paper chromatograms to determine their effectiveness in separating the "unknown" amino acid from the other amino acids present in the acid digest of yeast extract. *n*-Butanol-acetic acid-water 4:1:1 seemed best suited for this purpose (Fig. 2).

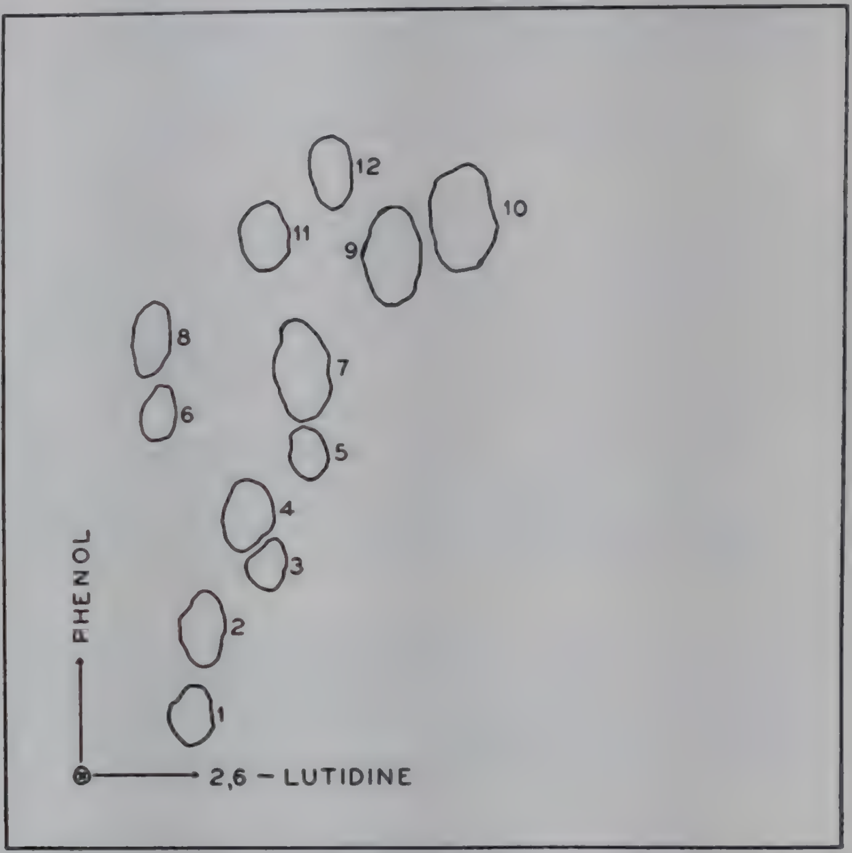

FIG. 1. Two-dimensional chromatogram of a chloride-free acid digest of yeast extract. The sample was applied at the lower left-hand corner and the solvents were allowed to move in the directions indicated, to the edges of the paper, as represented by the black lines in the figure. The phenol was saturated with water containing 10 per cent sodium citrate. (The use of sodium citrate was recommended by Helen Kirby Berry of this laboratory, to prevent distortion of the lower half of the developed chromatograms due to impurities in the phenol and possibly the filter paper.) The 2,6-lutidine contained water (65:35). Aspartic acid, *1*; glutamic acid, *2*; serine, *3*; glycine, *4*; threonine, *5*; lysine, *6*; alanine, *7*; arginine, *8*; valine, *9*; leucine or isoleucine, *10*; "unknown" amino acid, *11*; proline, *12*. The position of *11* was unchanged by prolonged acid hydrolysis of yeast extract.

Partition Chromatography on Starch—To 10 ml. of the chloride-free acid digest of yeast extract (containing approximately 1.13 gm. of solids) were added 2 to 3 gm. of potato starch,[3] and the mixture was lyophilized. To the dry powder were added several ml. of solvent to form a slurry and this was transferred to the top of a starch column (21 × 420 mm.) prepared in the usual manner from 115 gm. of starch. The column, with a solvent reservoir, was mounted on a Technicon automatic fraction collector,[4] and the effluent was collected in 5 ml. fractions. Separation of the amino acids on the column was traced by paper partition chromatography of samples of each fraction of the effluent. The order of emergence of the amino acids from the column was the same as the order of the band rates on paper chromatograms (Fig. 2). The unknown amino acid began to emerge from the column after approximately 250 ml. of effluent had been collected. The chromatogram was stopped when most of the alanine had

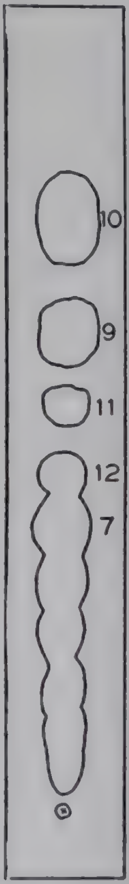

Fig. 2. One-dimensional chromatogram of a chloride-free acid digest of yeast extract. The sample was applied near the bottom of the paper and the solvent system (4:1:1 *n*-butanol-acetic acid-water) was allowed to move up the paper. The numbering of the amino acids is the same as in Fig. 1.

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[3] Obtained from the Fisher Scientific Company, Pittsburgh, Pennsylvania.

[4] Obtained from the Technicon Company, New York.

emerged from the column. All fractions containing the unknown amino acid, some of which were contaminated with valine and others with proline and alanine, were combined and evaporated to dryness *in vacuo.* The residue was dissolved in water and rechromatographed on a separate column according to the directions described above. From this second chromatogram, several fractions were obtained which contained the amino acid uncontaminated by other amino acids, as demonstrated by paper chromatography.

Isolation of Amino Acid—Five individual starch chromatograms were prepared and developed according to the procedure described above. All fractions containing the unknown amino acid were combined, evaporated to dryness *in vacuo*, and the residue was rechromatographed on a single starch column. Those fractions containing only the unknown amino acid, as revealed by paper chromatography, were combined and evaporated to dryness *in vacuo.* The gummy residue (about 90 mg.) was dissolved in 2 to 3 ml. of water and the solution was decolorized with Darco G-60 and evaporated to dryness *in vacuo.* The residue was dissolved in 2 drops of water and 2 to 3 ml. of hot ethyl alcohol were added. Crystals separated spontaneously from the solution. After two more recrystallizations, 11 mg. of crystals were obtained; m.p. 192–195° (uncorrected).[5]

Identification of Amino Acid—The crystalline sample was chromatographically pure. It possessed migration characteristics on paper chromatograms which were different from those of the known naturally occurring amino acids and a number of synthetic amino acids. Hydrolytic treatment did not affect the amino acid, as revealed by paper chromatography. The neutral equivalent obtained by electrometric titration was 102; N, 13.33 per cent. These data correspond to the empirical formula $C_4H_9O_2N$, an aminobutyric acid; N, calculated, 13.58; neutral equivalent, 103.1.

The infra-red absorption spectrum of the isolated amino acid was markedly different from that of α-amino-*n*-butyric acid, α-aminoisobutyric acid and β-amino-*n*-butyric acid[6] as shown in Fig. 3. An authentic sample of γ-aminobutyric acid, m.p. 193–196° (uncorrected),[5] was prepared according to the directions given in "Organic syntheses" (8). The hydrochloride of this sample melted at 132–133° (uncorrected). Abderhalden and Kautzsch report a melting point of 135–136° (9). The benzoyl derivative melted at 79° (uncorrected).[5] The melting point reported by Kanewskaja (10) is 75°; N 6.53 per cent; theoretical 6.76 per cent. The benzoyl derivative was converted to *N*-benzoylpyrrolidone, m.p. 91–92° (uncorrected).[5] An identical value was reported by Kanewskaja (10).

A comparison of the infra-red spectrum of the synthetic sample with

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[5] These are micro melting points.

[6] These three compounds were Eastman Kodak White Label products.

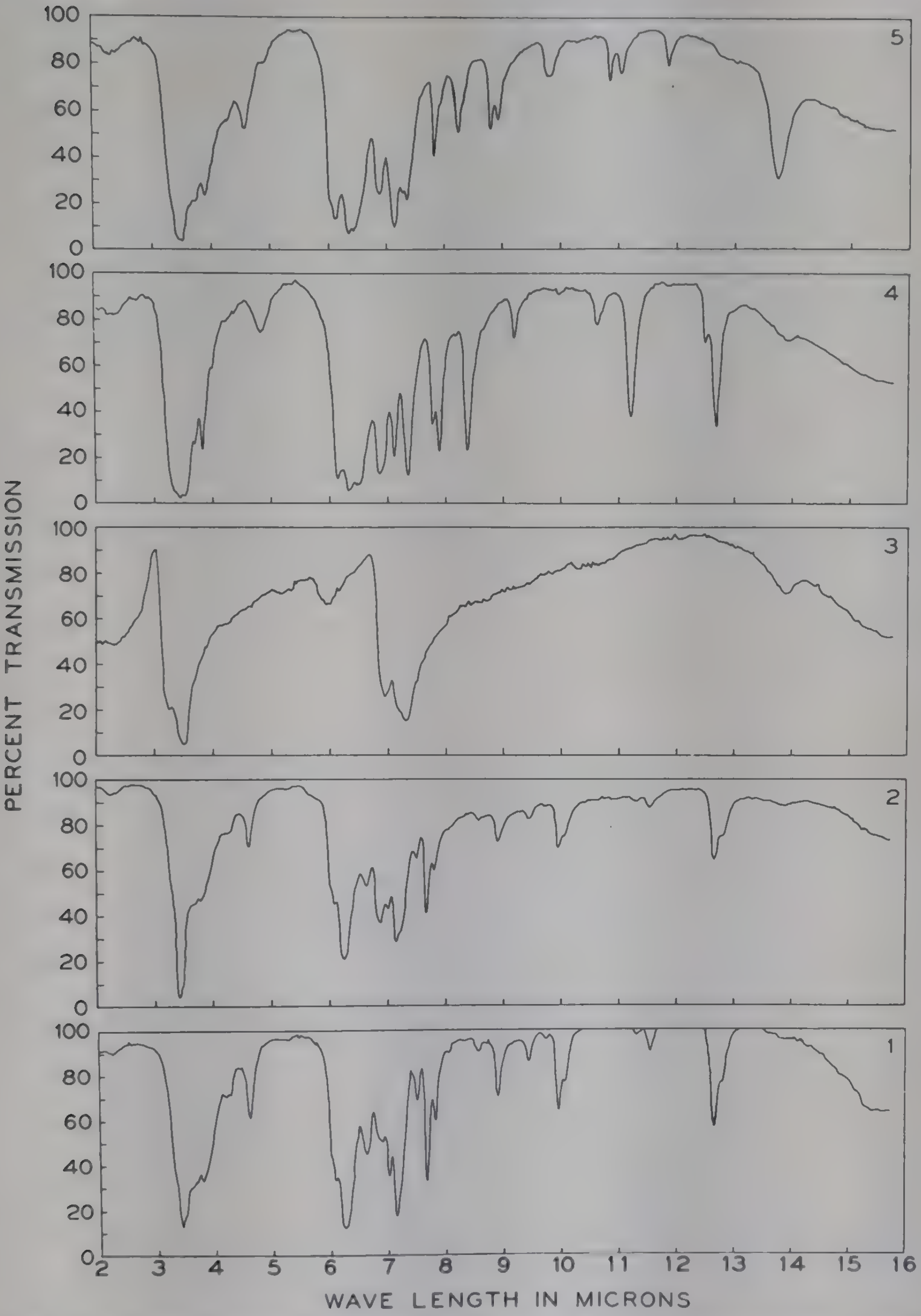

Fig. 3. Infra-red absorption spectra of isomeric aminobutyric acids. The samples were mulled in mineral oil. Spectra 1 and 2, natural and synthetic γ-aminobutyric acids, respectively; Spectrum 3, α-amino-*n*-butyric acid; Spectrum 4, α-aminoisobutyric acid; Spectrum 5, β-amino-*n*-butyric acid.

that of the natural amino acid indicated that the two compounds are identical (Fig. 3). Confirmation of this identity was obtained by comparing the x-ray diffraction patterns of the two samples (Fig. 4). Chromatographic evidence demonstrated further that the isolated amino acid

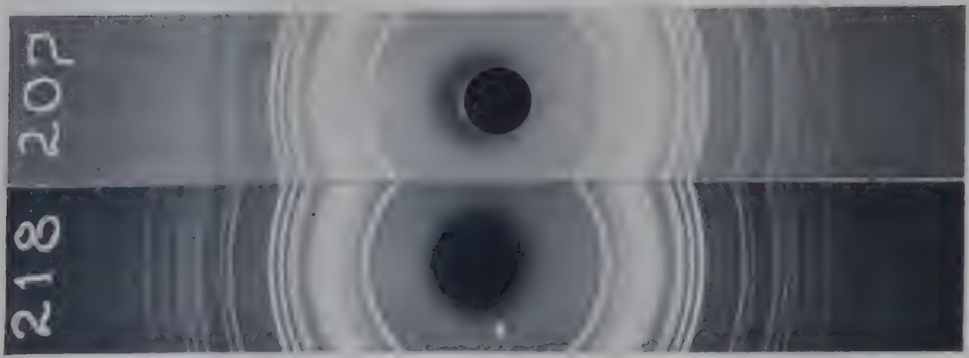

FIG. 4. X-ray diffraction patterns of natural (Sample 207) and synthetic (Sample 218) γ-aminobutyric acids.

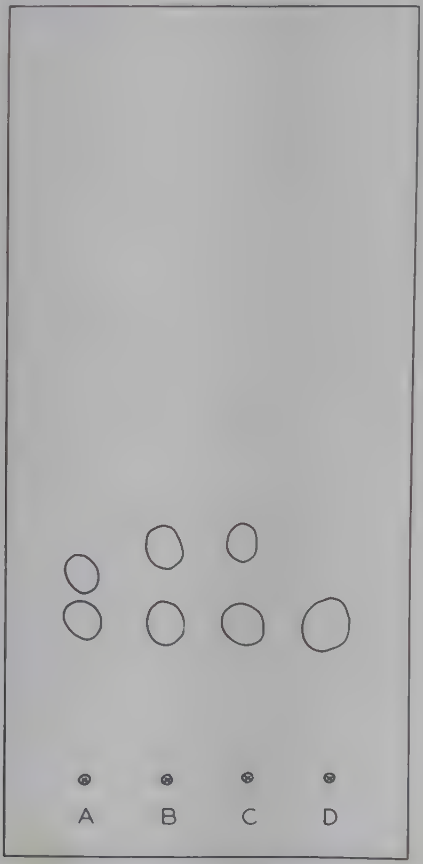

FIG. 5. One-dimensional chromatograms of mixtures of natural γ-aminobutyric acid and synthetic aminobutyric acids. Mixtures of the natural amino acid (3 γ) and each of the following synthetic aminobutyric acids were applied near the bottom of the paper: *A*, β-aminoisobutyric acid (4 γ) (prepared originally by Pollack (11) in this laboratory); *B*, α-amino-*n*-butyric acid (3 γ); *C*, α-aminoisobutyric acid (4 γ); *D*, γ-aminobutyric acid (3 γ). The solvent system was 2,6-lutidine-water. The lower spot in each case is the natural γ-aminobutyric acid.

and the synthetic γ-aminobutyric acid are identical (Fig. 5). β-Aminobutyric acid did not give a spot when tested in amounts up to 20 γ. Chromatograms developed with other solvent systems (phenol-water, *n*-butanol-acetic acid-water, isobutyric acid-water) revealed only a single spot when the sample chromatographed consisted of a mixture of the isolated amino acid and synthetic γ-aminobutyric acid.

Estimation of Amount of γ-Aminobutyric Acid in Yeast Extract—Further chromatographic experiments revealed that γ-aminobutyric acid is present in the free state in yeast extract (Difco). The amount of this amino acid was estimated by application of the method described recently by Bull and coworkers (12) for the quantitative determination of amino acids by filter paper chromatography. Known amounts of yeast extract, of synthetic γ-aminobutyric acid, and of mixtures of the two were run on a single large chromatogram with *n*-butanol-acetic acid-water as the solvent system (Fig. 2). The chromatogram was developed with ninhydrin, and color intensities were measured by means of a densitometer.[7] It was estimated that yeast extract (Difco) contains 1 to 2 per cent by weight of γ-aminobutyric acid.

DISCUSSION

The present investigation establishes the occurrence of γ-aminobutyric acid in yeast extract. Since completion of this work, chromatographic evidence has been presented (13) which indicates that γ-aminobutyric acid is probably present in traces in blood and urine and in potato extracts. It has been recognized for many years that this amino acid is produced by bacterial action on glutamic acid (14). The glutamic acid decarboxylase involved in this reaction has not been demonstrated yet to occur in other living cells.

Studies in this laboratory with yeast indicate that γ-aminobutyric acid is a degradative product. It was found to be absent in freshly harvested yeast, but present in the free state in whole yeast which had been refrigerated for unknown periods of time, and in autolysates of yeast. These studies are being continued and will be reported at a later date.

SUMMARY

Partition chromatography on paper indicated the presence of an amino acid of unknown nature in yeast extract (Difco) which had been subjected to mild acid hydrolysis. This amino acid was isolated from the acid-treated yeast extract and identified as γ-aminobutyric acid. It was demonstrated later to be present in the free state in untreated yeast extract to the extent of 1 to 2 per cent by dry weight.

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[7] Obtained from the Photovolt Corporation, New York.

The author is grateful to Mr. W. L. Brown for the elementary analysis and neutral equivalent; to Dr. S. F. Kern for the x-ray diffraction data; to Mr. T. V. Parke for the infra-red absorption data; and to Dr. E. Bottorff for the preparation of the authentic sample of γ-aminobutyric acid, all of Eli Lilly and Company. The technical assistance of Miss Patricia Johnston is gratefully acknowledged.

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DISTRIBUTION OF SELENIUM IN SERUM PROTEINS AND RED BLOOD CELLS AFTER SUBCUTANEOUS INJECTION OF SODIUM SELENATE CONTAINING RADIOSELENIUM

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In 1938, Smith, Westfall, and Stohlman (1) demonstrated that following prolonged feeding of naturally occurring seleniferous grains selenium is deposited in the body tissues, apparently in combination with the tissue proteins. Later work by Westfall and Smith (2), in which the distribution of selenium in liver and plasma of chronically poisoned animals was studied, showed that the element was present in all the proteins examined, though predominantly in the globulins. The manner in which the selenium in animal tissues is chemically bound is not known. A possible hypothesis is that selenium displaces the amino acid sulfur, or that its fixation involves the binding of sulfhydryl groups (3).

In earlier time-distribution studies the author observed a definite retention of trace amounts of radioselenium in the red blood cells and plasma of rats after subcutaneous injections of sodium selenate (4). The form in which the selenium enters the body apparently has little to do with its fixation.

In the experiments presented here it was planned to study the qualitative and quantitative distribution of selenium in the various blood proteins and in red blood cells. Following subcutaneous injection of sodium selenate containing trace amounts of radioselenium, the fixation of selenium in the proteins of plasma and in the hemin and globin fractions of hemoglobin was observed.

Procedure

Four normal dogs weighing 7.3 to 9.1 kilos were injected subcutaneously three times at 24 hour intervals with sublethal amounts of sodium selenate containing radioselenium. Previous to the second and third injections, and 24 hours after the last injections, samples of blood were taken from the carotid artery. Hematocrit and routine hematological studies were made. The red blood cells and plasma were separated by centrifugation, and the cells were washed twice with isotonic saline. Radioactivity measurements were made on samples of whole blood, plasma, and packed red blood cells, by use of a bell type Geiger-Müller tube.

The radioselenium was produced at Oak Ridge, Tennessee, by bombarding selenium powder with neutrons (5). The equation for the formation of radioselenium is as follows:

$$Se^{74}_{34} + N^{1}_{0} \rightarrow Se^{75}_{34} + \gamma$$

Se^{75} has a half life of 115 ± 5 days which decays through K electron capture, emitting a probable 0.4 m.e.v. γ-ray, with the formation of stable As^{75}_{33} (6).

The radioselenium was purified and sodium selenate was synthesized by methods described in a previous publication (4). Total selenium and radioactivity were determined on a sample of sodium selenate containing radioselenium. It was found that the sample contained 263 c.p.m. per microgram of selenium. This value was used throughout the experiments. A decay curve was constructed which showed the presence of a single isotope having a half life similar to that reported by Friedlander *et al.* (6).

For the purpose of studying the distribution of selenium in the various serum proteins, samples of blood were taken from the dogs 24 hours after the last injection. Total proteins were precipitated with 2.5 per cent trichloroacetic acid. Fractionation of the various serum proteins was carried out by the method of Majoor (7) as modified by Milne (8). Total globulins were precipitated from albumin with 26.8 per cent sodium sulfate solution, and euglobulins were separated from pseudoglobulins and albumin by use of 19.6 per cent sodium sulfate solution. The activity in the protein precipitates was measured, and the protein content of the precipitates and filtrates was determined by Clark's micro-Kjeldahl method (9). Selenium activity in the pseudoglobulin fractions was determined by subtracting that in the euglobulin fractions from that in the total globulins, and the activity in albumin was determined by subtracting that in the globulins from that in the total protein.

Hemoglobin and its hemin and globin fractions were studied to determine where the selenium was located in the red blood cells. Crystalline hemoglobin was isolated from the red blood cells of three dogs that had been injected with sodium selenate containing radioselenium, by use of the method of Welker and Williamson (10). The crystalline hemoglobin was recrystallized, and it was found that photomicrographs of the crystals compared favorably with those reported by Reichert and Brown (11). Crystalline hemin was isolated by the method of Nencki and Zaleski (12). The crystals were washed with 50 per cent acetic acid, 95 per cent alcohol, and ether. The globin fraction was studied following treatment of crystalline hemoglobin with acetone and hydrochloric acid after the method of Anson and Mirsky (13). The globin was dissolved in chilled 0.1 N hydrochloric acid and precipitated with acid acetone three times.

The various blood fractions were prepared for measurements of radioactivity by wet ashing with concentrated nitric acid and hydrogen peroxide. Digests were made to a 10 cc. volume, and 1 cc. aliquots were taken for counts.

A recovery study was made. The average counts in Table I with the standard error of 8.25 show no evidence that the presence of liver and bone tissue affects the determination of the activity of the standard. The average net count represents the average count per minute (minus background) obtained from two 5 minute determinations. Values for the original standard were obtained from an aliquot of sodium selenate containing radioselenium. For a control, an equivalent amount of radioselenium was added to the amounts of HNO_3 and H_2O_2 that were used for ashing tissues and was then carried through all the experimental steps. An equivalent amount of radioselenium was then added to various tissues which were wet ashed and the radioactivity determined.

TABLE I

Recovery of Radioselenium Added to Tissues

Standard error of each average count 8.25 counts per minute.

Tissue	Average net count
Original standard	197.3
Control (with all steps through reagents identical)	199.4
1 gm. normal liver + same amount standard	193.0
1 " " " + " " "	224.0
2 " " " + " " "	190.4
2 " " " + " " "	194.9
1 " " bone + " " "	205.1
2 " " " + " " "	207.7

RESULTS AND DISCUSSION

The data obtained in these experiments are presented below in three sections, dealing with the distribution of selenium (1) in the plasma and red blood cells, (2) in the various serum protein fractions, and (3) in the hemoglobin of the red blood cells.

Selenium in Plasma and Red Blood Cells—The distribution of selenium in dog plasma and red blood cells after repeated injections of sodium selenate containing radioselenium is shown in Fig. 1. The results are expressed as counts per 100 cc. of whole blood. The activity values for plasma and red blood cells were obtained by multiplying the number of counts for 100 cc. of plasma and red blood cells by the corresponding hematocrit value.

It will be noted that the concentrations of selenium in whole blood and in the red blood cells increase appreciably with time, and that the concentration is greater in the red blood cells than in the plasma. It appears that the concentration of selenium in the plasma when determined at 24 hour intervals varies within very narrow limits throughout the experiment.

Samples of blood were taken from Dog A 24 hours after the last injection, as well as at 48 and 144 hour periods. The results show that the selenium content of whole blood, red blood cells, and plasma decreases with time, the plasma values falling off rapidly to a very low level. The level for the red blood cells decreases but remains relatively high in relation to the plasma.

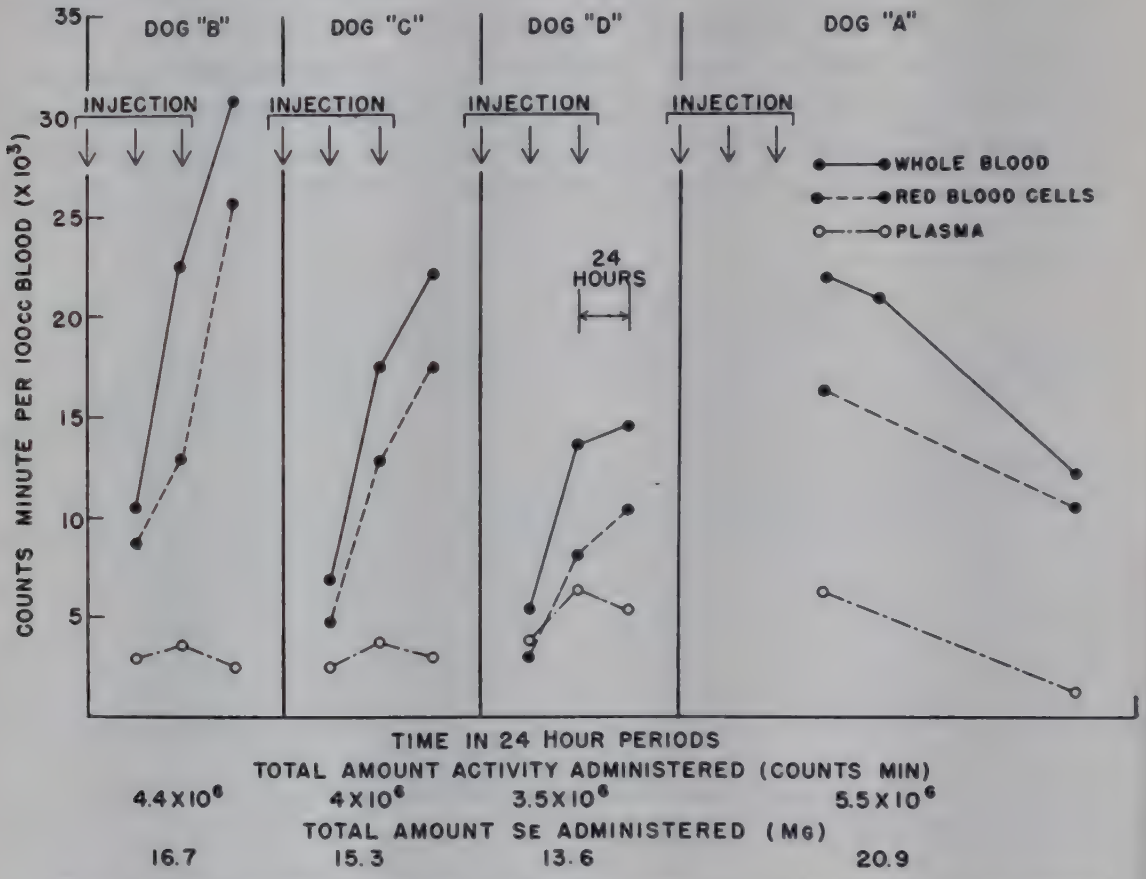

FIG. 1. Distribution of selenium in dog plasma and red blood cells after repeated subcutaneous injection of sodium selenate containing radioselenium.

It was of interest to determine the per cent of whole blood activity in red blood cells and plasma 24 hours after the last of three injections. The activity in the red blood cells was found to be 74, 91, 80, and 72 per cent (average of 79) for Dogs A, B, C, and D respectively. The activity in plasma was 26, 8, 14, and 39 per cent (average of 22), respectively.

By use of the value of 7 per cent of body weight as the total circulating blood (14), it is possible to calculate the per cent of the total selenium administered in the total circulating blood 24 hours after the last of three injections. The results are as follows: Dog A 2.5 per cent, Dog B 3.9 per cent, Dog C 3.4 per cent, and Dog D 2.0 per cent.

It is apparent from the data reported here that selenium is concentrated to a greater extent in the red blood cells than in the plasma, and that 2.0 to 3.9 per cent of the total selenium administered is found in the total circulating blood.

Selenium in Serum Proteins—In the next series of experiments, the distribution of selenium in the various serum protein fractions was studied. Table II shows the fractionation of serum proteins of three dogs into their various components; *i.e.*, total globulin, albumin, euglobulin, and pseudoglobulin. The selenium content in total protein ranges from 5.4 to 10.2 γ per gm. of protein. It will be noted that selenium is not found in any one fraction, but is present in all of the protein fractions examined, and the amount in each of the various fractions is in general similar but not identical. It appears that the fixation of selenium occurs to about the same extent in the globulin and albumin fractions, since the amount of globulin and albumin selenium with respect to total protein selenium for the three dogs was found to be 54 ± 10 and 45 ± 11 per cent, respectively.

TABLE II

Distribution of Selenium in Various Protein Fractions of Dog Serum after Subcutaneous Administration of Sodium Selenate

Fraction	Dog A		Dog B		Dog D	
	Counts per min. per gm. protein	Se per gm. protein	Counts per min. per gm. protein	Se per gm. protein	Counts per min. per gm. protein	Se per gm. protein
		γ		γ		γ
Total protein	2685	10.2	2278	8.7	1418	5.4
Globulin	2563	9.7	2614	9.9	1463	5.6
Albumin	3590	13.6	1672	6.4	1250	4.8
Euglobulin	1360	5.2	2434	9.2	1558	5.9
Pseudoglobulin	3530	13.4	3371	12.8	1255	4.8

The question arises whether the affinity of protein for selenium results in a definite fixation in the protein matrix or whether selenium in the protein fraction is due to adsorption on the surface of the protein molecule. The following information tends to support the contention that selenium occupies a well defined position in the protein molecule: Serum protein selenium in dilute solution resists thorough dialysis against neutral, acidic, and basic solutions. A sample of total serum protein was precipitated, and the precipitate washed thoroughly, dissolved in isotonic saline, and divided into four equal aliquots. One aliquot served as a control and was analyzed for total protein and radioselenium. The remaining aliquots were dialyzed against buffered solutions maintained at pH 2.8, 7, and 9, after which protein and selenium determinations were made. The results reveal that the selenium was not dialyzed under the above conditions (Table III).

The results indicate that the selenium present in the blood proteins is in some organoselenium complex. In this regard it is of interest to compare the metabolism of inorganic selenium with that of inorganic sulfur. It has been shown that inorganic sulfur can be converted to organic sulfur. Smythe and Halliday (15) were able to demonstrate the formation of cysteine from sulfide sulfur in the presence of a suitable liver enzyme. In another study, Dziewiatkowski (16) was successful in showing that a small fraction of the radioactive sulfur administered to rats as sulfide was incorporated in the cystine isolated from hair, liver, skeletal muscle, and skin. More recently, Dziewiatkowski (17) reports that labeled S^{35}-sodium sulfate, when administered to suckling rats, was found to be present in the articular cartilage as chondroitin sulfate. As we have proposed in an earlier report (18), a similar metabolic process may take place with selenium, as with sulfur, in the conversion of inorganic selenium to some organoselenium protein complex. Whether selenium displaces the amino acid sulfur or binds sulfhydryl groups or combines with an available radical cannot be stated at this time.

TABLE III

Dialysis of Dog Serum Proteins at Various pH Values

Clark and Lubs buffer mixtures were used.

Experiment	Se per gm. protein
	γ
Control	3.4
pH 2.8	4.0*
" 7.0	3.3*
" 9.0	3.8

* Corrections made for potassium activities.

Selenium Activity in Hemoglobin, Hemin, and Globin—In the study on the distribution of selenium in the various components of the red blood cell, hemoglobin, hemin, and globin were isolated and analyzed separately for selenium activity. Data obtained from these studies are presented in Table IV, where weighed samples of crystalline hemoglobin and hemin, and of globin were found to have 4.5 to 5.7, 10.6 to 15.5, and 1.3 to 2.4 γ of selenium per gm. respectively. Activity ratios of hemin to globin on the gm. basis for two dogs were found to be 6.3 and 8.1. It is apparent from the data presented here that selenium is present in hemoglobin, and that the amount of selenium in the hemin fraction when calculated on the gm. basis is greater than that in the globin fraction.

The fixation of selenium in red blood cells presumably does not take place in bone marrow, for we were unable to detect any significant selenium activity in bone marrow 24 hours after the last of three injections. The incorporation of selenium in hemoglobin may take place by exchange with some normally occurring substance, or by addition to an available radical after diffusion across the cell membrane. Inorganic selenium is quite unlike radioactive iron in that iron is synthesized into hemin in the bone marrow (19). Of particular interest is the apparent difference between selenium and sulfur in their ability to concentrate in the bone marrow.

TABLE IV

Selenium Activity of Hemoglobin, Hemin, and Globin in Dogs after Subcutaneous Administration of Sodium Selenate

Dog	Activity of hemoglobin		Activity of hemin		Activity of globin		Ratio, hemin-globin, gm. basis
	Counts per min. per gm. Hb	Se per gm. Hb	Counts per min. per gm. hemin	Se per gm. hemin	Counts per min. per gm. globin	Se per gm. globin	
		γ		γ		γ	
B	1449	5.50	4082	15.5	644	2.4	6.3
	1506	5.72	4016	15.3			
C	1235	4.69	2786	10.6	346	1.3	8.1
	1329	5.05					
A	1225	4.65					
	1184	4.50					

Singher and Marinelli (20) as well as Dziewiatkowski (21) were able to demonstrate, using labeled sodium sulfate, that S^{35} was found in the bone marrow, while we were unable to detect any selenium activity in dog bone marrow or in rat bone (4) after administering labeled sodium selenate. The destruction and elimination of selenium-bound hemoglobin may take place, in part, in the liver, for we have observed the selenium content in bile 24 hours after the last of three injections in three dogs to be 4.2, 8.6, and 4.3 γ of selenium per cc. of bile.

SUMMARY

Studies were made on the distribution of selenium in the blood, serum proteins, and red blood cells of the dog after subcutaneous injections of radioselenium as sodium selenate.

1. It was found 24 hours after the last of three injections that the whole

blood activity in red blood cells for four dogs averaged 79 per cent (72 to 91 per cent), while in plasma it averaged 22 per cent (8 to 39 per cent). It was calculated that 2.0 to 3.9 per cent of the selenium administered was present in the total circulating blood.

2. The results obtained from serum protein fractionation revealed that selenium was present in albumin, globulin, euglobulin, and pseudoglobulin, and that the amount of selenium in the various fractions when expressed as micrograms of selenium per gm. of protein was similar but not identical. Results from dialysis studies indicated that there was a certain amount of selenium fixation in blood proteins which existed in a fairly stable protein-like combination.

3. It has been shown that selenium is present in crystalline hemoglobin and hemin and in globin. The amounts of selenium in hemin when calculated on the gm. basis are greater than in globin.

The authors wish to express thanks to Dr. Joe W. Howland for his interest in this investigation.

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BIOLOGICAL ACTIVITY OF PURE VITAMIN A_2*

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The degree of biological activity for mammals that can be attributed to vitamin A_2 has long been a matter of disagreement among various investigators. Karrer *et al.* (1) first reported that vitamin A_2 possessed no vitamin activity for mammals. This conclusion, however, was based on the difficult procedure of attempting to evaluate a mixture of vitamins A_1 and A_2 biologically. In a later paper (2) this same group claimed that vitamin A_2 had one-tenth the growth-promoting power of vitamin A_1 for rats and ascribed this activity to the ability of the animals to convert a small portion of the vitamin A_2 to vitamin A_1. These observations were based on biological tests carried out by Professor H. von Euler in Stockholm. On the other hand, Gillam *et al.* (3) believed that vitamin A_2 possessed a considerable degree of biological activity and showed (4) that vitamin A_2 was stored in the livers of animals receiving vitamin A_2 in their diet. Lederer and Rathmann (5) also demonstrated that vitamin A_2 could be stored in the livers of rats and frogs but the degree of biological activity was not determined. From a study of the vitamin A_1 and A_2 distribution in various fish and sea birds, Lovern *et al.* (6) concluded that vitamin A_2 does not replace vitamin A_1 with equal readiness for all functions.

Rosanova (7) states that vitamin A_2 has an "activating effect" upon the growth of rats and is a factor in their vision. Shantz *et al.* (8) found that in rats fed vitamin A_2 rhodopsin in the retinas was replaced by porphyropsin, and from this and other observations concluded that vitamin A_2 could replace vitamin A_1 in all physiological functions. Using human subjects, Millard and McCann (9) have shown that the threshold of rod sensitivity to red light can be lowered slightly by administration of vitamin A_2 over a period of 16 weeks.

In an earlier paper from this laboratory (10), a vitamin A_2 concentrate was reported to have a potency of 47,500 U. S .P. units per gm. Various reviewers have mistakenly reported that this potency referred to pure vitamin A_2 rather than to a concentrate of unknown strength (11–13). Actually, since the $E^{1\%}_{1\,\mathrm{cm.}}$ (351 mμ) value of the concentrate equaled 39.0, the conversion factor for vitamin A_2 could be calculated as $47{,}500 \div 39 =$

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1218, but no estimate could be made of the potency of pure vitamin A_2 because its $E^{1\%}_{1\,\text{cm.}}$ (351 mμ) value was not known.

The recently reported isolation of pure vitamin A_2 (14) in this laboratory and the acceptance of the new and stable oil solution of crystalline vitamin A acetate as the U. S. P. reference standard (15) have made possible a more accurate determination of the true biological potency of vitamin A_2.

EXPERIMENTAL

Biological Assay—The pure vitamin A_2 alcohol was biologically assayed by essentially the method of the United States Pharmacopœia XIII (15). The crystalline *p*-phenylazobenzoate of vitamin A_2 was also assayed. Two levels of vitamin A acetate reference oil, two levels of vitamin A_2 alcohol, and one level of vitamin A_2 *p*-phenylazobenzoate were fed to the rats. The growth response for each of these levels is shown in Table I.

TABLE I

Growth Response Produced by Vitamin A_2

No. of rats	Material fed	Amount fed	Weight gain in 28 days
		U. S. P. units	*gm.*
7	Vitamin A acetate reference standard	1.2	35.7
7	" " " " "	2.4	73.0
		mg.	
5	" A_2 alcohol	0.65	21.0
7	" " "	1.30	52.1
7	" " *p*-phenylazobenzoate	2.3	52.1

Curves of log dose *versus* growth response were plotted for the two levels of reference standard and the two levels of vitamin A_2 alcohol. Calculation by factorial analysis of variance according to Bliss and Marks (16) showed that each of the dosage levels of vitamin A_2 was equivalent to 70.0 ± 8.0 per cent of its corresponding level of reference standard. Thus, by this assay, vitamin A_2 was found to have a biological potency of 1,300,000 U. S. P. units per gm. and the true value probably does not deviate more than 100,000 units from this figure. Vitamin A_2, therefore, has approximately 40 per cent of the activity of crystalline vitamin A_1.

The vitamin A_2 *p*-phenylazobenzoate, which was fed at only one level, gave exactly the same growth response as the higher level of vitamin A_2 alcohol. Its potency was calculated to be 730,000 U. S. P. units per gm. for the crystalline ester. When only the vitamin moiety of this compound is considered, a value of 1,270,000 U. S. P. units per gm. is obtained, which is in excellent agreement with the value found for the free alcohol.

Conversion Factors—It is common practice to evaluate the potency of an oil containing vitamin A by multiplying its ultraviolet extinction coefficient by a conversion factor. From the above biological potency and the value of 1460 reported (14) as the extinction coefficient of pure vitamin A_2 alcohol at its absorption maximum at 351 mμ, it can be calculated that this compound has a conversion factor of approximately 900 when measured at this wave-length.

The extinction coefficient of vitamin A_2 in the vitamin A_1 region at 325 mμ is 80 per cent of its peak value, since its absorption curve is rather broad (14). At 325 mμ, therefore, the conversion factor for vitamin A_2 is $900 \div 0.8 = 1125$. Thus, if vitamin A_2 happens to be an unsuspected component of a fish liver oil, the true biological potency of the oil will tend to be slightly lower than that calculated from the observed spectrophotometric value at 325 mμ, with the currently accepted conversion factors of 1894 for vitamin A_1.

The broad overlapping of the vitamin A_1 and A_2 spectral curves (14) makes measurement of ultraviolet absorption a rather poor method of determining the relative quantities of each in mixtures. Morton and Stubbs (17–19) have proposed a correction procedure whereby it is claimed that the true E value of vitamin A in an oil can be determined by subtraction of the irrelevant absorption due to extraneous material. The distorting effect of interfering substances is calculated from the interrelationships of three reference points. These reference points are obtained from the absorption curve of pure vitamin A in a given solvent and occur at those wave-lengths at which the absorption is at a maximum and on either side when the absorption is 6/7 maximal. For the derivation of the formulas and their geometric basis, the reader is referred to the original papers.

The above correction method is based on two premises: first, that substances other than vitamin A have a linear absorption between the two "fixation points" on either side of the maximum, and second, that these substances have no vitamin A activity of their own. Neither of these assumptions holds true in the case of vitamin A_2.

Oser (20) has combined and simplified the correction factors of Morton and Stubbs into the single equation

$$f = 7 - 7a\frac{E_x}{E_m} - 7(1 - a)\frac{E_y}{E_m}$$

where f is the factor by which the observed E_m is multiplied to obtain the corrected value; E_m, E_x, and E_y are the observed extinction values at the wave-lengths m, x, and y respectively; and m, x, and y are those wave-lengths on the absorption curve of pure vitamin A_1 at which the maximum and the two "fixation points" below and above the maximum occur. The value $a = (y - m)/(y - x)$.

According to Oser (20) the values m, x, and y for vitamin A alcohol in ethanol solution are 325, 311, and 335.5 mμ, respectively, and the value thus obtained for $7a = 3.0$ and for $7(1 - a) = 4.0$. From an absorption curve of pure vitamin A_2 alcohol in ethanol solution, we have determined in this laboratory that the extinction ratio E (311 mμ) to E (325 mμ) = 0.69, and the ratio E (335.5 mμ) to E (325 mμ) = 1.15. By substituting in the above equation, it can be calculated that $f = 0.33$ when pure vitamin A_2 is measured as vitamin A_1 alcohol at 325 mμ, corrected by the Morton-Stubbs procedure. By this method, then, an oil which contains a significant amount of vitamin A_2 will tend to be overcorrected because that portion of the 325 mμ absorption which is contributed by vitamin A_2 will in effect be measured with a conversion factor of $0.33 \times 1894 = 625$ instead of the true factor of 1125. Such an error might be appreciable in certain commercial fish liver oils, which have been found to contain as much as 10 per cent of the vitamin A in the form of vitamin A_2 (19). In such a case the vitamin A_2 content of the oil might best be determined independently by calculation from the blue color absorption obtained with antimony trichloride with the E(620) to E(690) ratios suggested by Jensen *et al.* (10), or by the procedure of Embree and Shantz (21) in which the two anhydro derivatives are chromatographically separated.

TABLE II

Efficiency of Liver Storage of Vitamin A_2

Eight rats were fed 1500 U. S. P. units of material in 3 days in each test.

Material fed	Total amount per rat stored in liver	Per cent total dose stored
	U. S. P. units	
Vitamin A_1 alcohol	491	32.7
" " acetate	690	46.0
" A_2 alcohol	275	18.3
" " acetate	345	23.0

Liver Storage Tests—Four groups of vitamin A-depleted rats were fed biologically equivalent doses of the compounds listed in Table II for a period of 3 days. The animals were killed on the 5th day and the total amount of vitamin A stored in the liver was determined in each case. From an examination of Table II, it can be seen that the storage of vitamin A_1 is better than that of vitamin A_2, although within each group somewhat more of the acetate ester is stored than is the free alcohol. All of the test substances were diluted to approximately the same level (5000 U. S. P. units per gm.) in Wesson oil before feeding.

It is interesting to note that the liver storage ratios of vitamins A_1 and A_2 have roughly the same relationship as their biological potencies in the U. S. P. assay. However, it is difficult to say at the present time whether these findings are significant and related.

Non-Conversion of Vitamin A_2 in Vivo—Animals that were maintained on doses of vitamin A_2 close to the minimum daily maintenance level did not store enough vitamin in the liver for colorimetric determination. However, an excellent curve for blue color could be obtained with antimony trichloride on the liver extracts from rats receiving about 50 U. S. P. units per day. The curves thus obtained were identical in shape with that of the pure vitamin A_2 originally fed. This confirms our earlier report (9) that vitamin A_2 is not converted to vitamin A_1 in the animal body.

SUMMARY

Pure vitamin A_2 alcohol has a potency of about 1,300,000 units per gm. as determined by the U. S. P. rat growth procedure. It is stored in the liver with an efficiency somewhat lower than that of vitamin A_1, but does not appear to be converted to vitamin A_1 *in vivo*.

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PARTIAL PURIFICATION OF A FACTOR ESSENTIAL FOR GROWTH OF LEUCONOSTOC CITROVORUM

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Based on the growth response of *Leuconostoc citrovorum* to commercial antianemia liver preparations, Sauberlich and Baumann (1) suggested that the organism might be responding to the antipernicious anemia principle. Since vitamin B_{12} was available to us at that time, it was tested for its growth-promoting activity on this organism and was found to be negative. Efforts were then undertaken to concentrate and isolate the factor essential for the growth of this organism. Methods for the preparation of concentrates of an acid-labile factor and a description of some of its properties are now presented.

Methods

A culture of the organism, *L. citrovorum* (8081) was obtained from the American Type Culture Collection. It was maintained in yeast extract-glucose agar stabs and transferred in this medium at biweekly intervals. For assay, the organism from the stab culture was inoculated into a liver broth of the following composition:[1] tryptone, Difco, 2 per cent; tryptose, Difco, 0.5 per cent; yeast extract, Difco, 0.5 per cent; tomato juice, 20 per cent; water-soluble liver (Wilson), 0.5 per cent; glucose, 0.3 per cent; lactose, 0.2 per cent; Tween 80, 0.005 per cent, pH 6.5.

After incubation for 24 hours at 37°, the culture was washed twice in NaCl solution (0.85 per cent) and diluted 10-fold. 1 drop of the washed suspension in saline was used to inoculate each assay tube.

The assay medium was essentially that of Snell, Kitay, and McNutt (2). It was modified by the omission of Tween 80 and oleic acid and by doubling the recommended casein hydrolysate concentration. Difco casamino acids, untreated by charcoal, were used as the source of casein hydrolysate.

The above basal medium (double strength) was employed in a volume of 5 ml. to which a total of 5 ml. of water and sample was added. The tubes were autoclaved for 10 minutes at 10 pounds steam pressure, then placed in a cold water bath, shaken to dissolve a precipitate which formed, and cooled to room temperature. The assay tubes were then inoculated and incubated for 15 to 16 hours at 37°. Turbidity measurements were then made by means of an Evelyn photelometer with the 660 mμ filter.

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[1] Suggested by M. Rogosa.

A hot aqueous extract of fresh kale, containing 7.4 mg. of solids per ml., was initially employed as a reference standard. Later, an ammonium salt of the active material obtained after chromatography was substituted for this extract. Results are expressed in growth equivalent to the activity of the kale extract and 1 unit was arbitrarily established as representing the activity of 1 ml. of this extract.

Folic acid determinations[2] were made in the medium of Teply and Elvehjem (3).

EXPERIMENTAL

4 kilos of liver fraction S (Wilson) were suspended in 340 liters of water. The pH of the suspension was adjusted to 5.5 with sodium hydroxide or sulfuric acid as required. The insolubles were removed by filtration.

To this extract were added 4.5 kilos of norit A (Pfanstiehl), and it was stirred for 30 minutes. The norit adsorbate was recovered by filtration and washed with water. About 10 per cent of the activity was lost in the filtrate from the norit.

The activity was recovered from the norit by extracting three times with the following mixture: 13.3 liters of 95 per cent ethyl alcohol, 26.6 liters of water, and 1 liter of 10 per cent sodium hydroxide. The extractions were made at room temperature, with 30 minutes stirring. The combined eluates contained approximately 65 per cent of the original activity.

Readsorption—The combined eluates were acidified to pH 4.5 with 50 per cent sulfuric acid and stirred for 30 minutes with 1.5 kilos of norit A. The recovered norit adsorbate was washed with 5 liters of 30 per cent ethyl alcohol. It was then extracted four times with 15 liters of 30 per cent ethyl alcohol to which were added 750 ml. of concentrated ammonium hydroxide. The extractions were made at room temperature with vigorous stirring for 30 minutes.

The combined eluates contained 50 to 60 per cent of the original activity, and represented a 20-fold purification.

Butanol Extraction—The combined eluates were concentrated at reduced pressure, temperature 45–50°, to approximately 1 liter. This was then acidified to pH 2.3 with 10 per cent nitric acid. The precipitates which had formed during the concentration and acidification were removed by filtration through a thin layer of Super-Cel. About 10 per cent of the activity was retained in this precipitate. The acid filtrate was immediately extracted with approximately 12 liters of water-saturated butanol. The water phase was removed and contained about 15 per cent of the activity. The butanol layer was extracted five times with 8 liter portions of water. The aqueous extracts of the butanol which contained 30 to 35 per cent of the original activity were promptly neutralized with barium hydroxide and concentrated *in vacuo* at 45–50° to a volume of 150 to 200 ml. This concentrate was stored at 3–5° for several days, during which time a heavy precipitate formed which was removed and washed by centrifugation.

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[2] In the text folic acid is referred to as FA and *Streptococcus lactis* R as SLR.

The clear solution was then acidified to pH 4.9 with 10 per cent nitric acid. Silver nitrate was added in excess. The precipitate was recovered by centrifugation and washed with water. The washed silver precipitate was suspended in excess dilute ammonia water, and then the silver was precipitated as the sulfide with hydrogen sulfide. The silver sulfide was washed several times with water. It was found necessary to use ammonium hydroxide to maintain alkaline conditions during the decomposition of the silver precipitate, since acidity resulting in the decomposition of the silver precipitate with hydrogen sulfide was sufficient to inactivate a substantial portion of the growth factor.

The filtrate and washings resulting from the decomposition of silver precipitate were concentrated *in vacuo*. Excess barium hydroxide was added and the extract was further concentrated to about 75 ml. The recovery to this stage was 25 to 35 per cent.

The concentrate was then stored for several days at 3–5°. The insoluble material which separated was discarded after washing with 30 to 40 ml. of water. The clear extract and wash were combined and 4 volumes of 95 per cent ethyl alcohol were added. This was set in the refrigerator overnight. The precipitated barium salts were recovered, washed with alcohol, acetone, and ether, and finally dried *in vacuo*.

At this stage the recovery of activity was 20 to 30 per cent. The potency of the barium salts varied from 70 to 300 units per mg., which corresponded to a 70- to 300-fold purification of starting liver fraction S.

Chromatography—To effect further purification the barium salts were chromatographed on aluminum oxide. The adsorptions were carried out in acid alcohol and elutions by dilute ammoniacal aqueous alcohol. A typical example is as follows: 14.42 gm. of a barium salt having a potency of 109 units per mg. were dissolved in 200 ml. of water. 10 per cent hydrochloric acid was added to pH 2.3. 3 volumes of 95 per cent ethyl alcohol were added. The precipitate which formed was separated by centrifugation and washed with 400 ml. of 75 per cent ethyl alcohol. The main solution and wash were combined and allowed to pass through a 40 mm. column containing 300 gm. of aluminum oxide (Alcoa). The column was washed with 30 per cent ethyl alcohol and eluted with 30 per cent ethyl alcohol to which was added 1 per cent concentrated ammonia water. Fractions were taken as indicated in Table I.

TABLE I

Distribution of L. citrovorum Activity on Aluminum Oxide Column

Fraction No., 50 ml.	Activity	Potency	Fraction No., 50 ml.	Activity	Potency
	units	*units per mg.*		*units*	*units per mg.*
7*	1,800	24	15	144,000	3200
8	12,600	210	16	117,000	3340
9	45,500	600	17	72,000	2400
10	99,000	1320	18	49,000	2450
11	166,500	2875	19	33,000	940
12	190,000	3450	20	29,500	1060
13	187,500	3410	21	26,000	650
14	158,000	3160			

* Fractions 1 through 6 contained 695 mg. of solids but no activity.

Acid Lability and Other Properties of Concentrates—Concentrates active for the test organism show marked acid lability. The effect of storage for 6 hours at room temperature at several pH values is as follows:

pH, adjusted with H_2SO_4	1.12	1.30	2.20	3.05	3.60	4.18	4.72	5.0
Loss, %	100	100	81	30	13	0	0	0

Loss of growth activity is evidenced at pH 3.6. In 0.1 N H_2SO_4, 90 to 100 per cent destruction results in 24 hours at room temperature or in 15 minutes when the material is autoclaved at 10 pounds steam pressure. No destruction under such conditions is detectable when the active material is stored in 0.1 N NaOH.

The distribution of the *L. citrovorum*-active material between water and an equal volume of water-saturated normal butanol is dependent on pH. The effect of pH on the distribution is as follows:

pH	5.83	4.69	3.80	3.27	2.30
Activity in butanol layer, %	1.0	9.5	33.0	40.0	48.5

An equal distribution of the activity between the two phases occurs at pH 2.2 to 2.3. Lyman and Prescott (4) by electrolytic procedures have also indicated the acidic properties of the *citrovorum* factor.

The active material is completely precipitated by heavy metals such as lead and silver. Only partial precipitation (40 to 50 per cent) occurs at pH 7.0 with zinc. At pH 2.3 the material is not extracted by ethyl ether or chloroform and is not precipitated from aqueous solution by ethanol or acetone. Esterification procedures (diazomethane or methanol and HCl) resulted in complete inactivation as indicated by lack of activity after alkaline hydrolysis. Acylation also resulted in inactivation.

Relationship of SLR to L. citrovorum Activity—Concentrates containing the *citrovorum* factor are also active in promoting the growth of *S. lactis* R and *Lactobacillus casei* in media lacking folic acid. This relationship is indicated for SLR in Table II, showing the activity of successive samples obtained during chromatography. The growth activity for SLR paralleled that for *L. citrovorum*. This is indicated by the relatively constant ratios shown.

TABLE II

Ratios of SLR to L. citrovorum Activity

Sample No.	SLR*	*Citrovorum*	$\frac{\text{SLR}}{\textit{citrovorum}}$	SLR after acid treatment	Per cent residual SLR activity
	mμgm per ml.	*units per ml.*		*mμgm. per ml.*	
1	200	35.2	5.7	108	54
2	8,900	1480	6.0	4,800	54
3	25,800	4800	5.4	13,950	54
4	28,750	4900	5.9	15,000	52
5	27,000	4920	5.5	13,950	52
6	19,200	3330	5.7	11,800	59

* SLR activity reported in terms of FA.

Acid treatment of these fractions also results in a rather consistent percentage loss in SLR growth activity (Table II). As indicated previously, the *citrovorum* factor is acid-labile and storage at room temperature (24 hours) in 0.1 N HCl, which produces 90 to 100 per cent loss for *L. citrovorum*, resulted in 41 to 48 per cent loss of SLR activity. Storage of FA under similar conditions results in no loss of activity for SLR. The implications of these results are considered in the discussion.

Sauberlich (5) has shown that rats fed FA excrete increased amounts of *citrovorum*-active material in urine. This suggests the *in vivo* conversion of FA to *citrovorum* factor. In limited trials in humans, 25 mg. of FA taken orally led to the excretion of but 2000 to 3000 *citrovorum* units in 24 hours. Assuming that the conversion of FA to the *citrovorum* factor results in no substantial change in molecular weight, from the tentative maximal activity ratio of SLR to *citrovorum* of 5, the 3000 *citrovorum* units are equivalent to only 0.015 mg. Thus if the *citrovorum* factor is a conversion product of FA, less than 0.1 per cent of the ingested dose was recovered in the urine in such form.

To check further on a possible relationship of the *citrovorum* factor to FA, a comparison was made of the rate of liberation of the two activities during the autolysis of normal rat liver. A fresh rat liver was minced in a Waring blendor and autolyzed at 37°. Samples were taken at the intervals indicated in Table III. The data show that the two activities are liberated at different rates and that the *citrovorum*-active material appears to be released independently from that for SLR.

TABLE III

Liberation of SLR and L. citrovorum Activity from Rat Liver

Digested	SLR*	*Citrovorum*	$\frac{\text{SLR}}{\textit{citrovorum}}$
hrs.	*mμgm. per gm.*	*units per gm.*	
0	835	5.0	167
2	2237	52.0	43
4	2220	58.5	38
21.5	2337	149.0	16
24	2160	162.0	13

A 17 gm. rat liver was minced in a Waring blendor for 1½ minutes at 0° in 0.03 M phosphate-citrate buffer at pH 7.0. The suspension, under toluene, was then incubated at 37°.

* Reported in terms of FA activity.

DISCUSSION

The data concerning the parallelism of SLR and *L. citrovorum* activity (Table II) are open to several interpretations. It seems likely at this time that the most highly purified concentrates contain two active substances, FA which is active for SLR only, and the *citrovorum* factor which is active also for SLR. On acid treatment of the *citrovorum* factor, activity for both organisms is lost. The residual SLR activity is due therefore to original contamination with FA.

On the other hand if the FA content of the concentrates is insignificant, then the *citrovorum* factor on acid degradation is converted to FA. The question as to which of these two hypotheses is correct must await the further purification of *citrovorum* factor.

In this connection it is of interest that Pfiffner *et al.* (6) have reported the occurrence in liver of an acid-labile material having FA activity for SLR. In view of the similar procedures employed in this concentration it seems likely that their acid-labile factor and the *citrovorum* factor are the same, or closely related.

SUMMARY

A method is described for the preparation of concentrates of the factor required for growth of *Leuconostoc citrovorum* 8081. Some of the properties of such concentrates are described. Preparations active for *L. citrovorum* were also active for *Streptococcus lactis* R.

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THE PRELIMINARY CHARACTERIZATION OF TWO BIOTIN-CONTAINING FRACTIONS IN BEEF LIVER*

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A large fraction of the biotin in animal tissues has been *assumed* to be firmly bound to the tissues, and treatments known to destroy proteins such as acid hydrolysis or enzymatic digestion have been used routinely to liberate the vitamin from these materials for microbiological assay (1). Recently a study on "soluble bound forms" of biotin has been presented (2), and the isolation of a crystalline biotin complex (biocytin) active for *Saccharomyces cerevisiae* but not for *Lactobacillus arabinosus* has been reported (3). The present study was directed towards the isolation and characterization of biotin-protein complexes from animal tissues, since it seems likely that they represent the enzymatically active forms of biotin. Little information is available on the extractability from tissues of microbiologically inactive biotin-containing proteins. The presence of high molecular weight "saline-soluble" biotin complexes in egg yolk has been reported by György and Rose (4) who found that these materials although non-dialyzable had the same growth-promoting activity for yeast before and after hydrolysis with sulfuric acid.

Beef liver was selected as the starting material for the present investigation, since it is well established that this tissue contains biotin in a form which prior to hydrolysis is not available to microorganisms. The liver was dehydrated with acetone and the resulting acetone liver powder extracted under mild conditions. The resulting extracts were assayed by the yeast growth method for their biotin content before and after hydrolysis with acid. By the use of this technique it was found that beef liver contains at least two distinct biotin-containing fractions. One of these is extractable with a buffer at pH 3 and loses its biotin activity on dialysis; the other is soluble in dilute sodium hydroxide and is not affected by dialysis. The substance soluble at pH 3 has practically the same biotin activity before and after acid hydrolysis, in contrast to the alkali-soluble compound which has very little activity initially but becomes highly active following hydrolysis with sulfuric acid.

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EXPERIMENTAL

Preparation of Liver Powder—Fresh beef liver was obtained from a slaughter-house and immediately cooled with ice. The liver was ground in small portions in a Waring blendor with ice-cold acetone and the slurry filtered with suction on a Büchner funnel. The resulting material was reground with acetone, filtered, dried at room temperature for 2 to 3 hours, and finally stored in the refrigerator. From 500 gm. of fresh liver, 140 gm. of liver powder were obtained. Samples of the liver powder were autoclaved with 6 N sulfuric acid for 1 hour at 18 pounds pressure for total biotin determination. The biotin content of several preparations ranged from 3.3 to 4.5 γ per gm. (*S. cerevisiae* assay).

Preparation of Extracts—The liver powder was extracted with distilled water, universal buffer solutions (5) (ranging from pH[1] 3.0 to 11.0), and 0.025 N sodium hydroxide. 2 gm. samples of the liver powder were weighed into 50 ml. centrifuge tubes and each sample extracted three times with 25 ml. portions of the respective solvent by shaking overnight at 5° in a cold room. The resulting suspensions were separated by centrifugation and the extracts from each liver sample combined (approximately 75 ml.) and made up to 100 ml. with distilled water.

The dialyzed samples were obtained by dialyzing the above extracts in Visking sausage casing against running distilled water for 48 hours at 5°.

Small aliquots (1 to 2 ml.) of the extracts were autoclaved with 5 ml. of 6 N sulfuric acid for 1 hour at 18 pounds pressure for the preparation of the acid-hydrolyzed samples. The samples were adjusted to pH 4 with dilute sodium hydroxide and suitable dilutions used for assay.

Microbiological Assay Procedure—*S. cerevisiae* 139 was used as the test organism throughout this study. The assays were carried out according to the procedure of Snell, Eakin, and Williams (6) as modified by Hertz (7).

The assay flasks containing the culture medium were sterilized by autoclaving and cooled. Suitable dilutions of the extracts were then added and the flasks inoculated. The organisms were grown in 50 ml. Erlenmeyer flasks at 30°; growth was measured turbidimetrically by the use of a Klett-Summerson photoelectric colorimeter.

RESULTS AND DISCUSSION

The extraction of acetone liver powder with buffers, dilute sodium hydroxide, or water led to the results illustrated in Fig. 1. It may be noted that the growth-promoting activity of the resulting extracts decreased with increasing pH (Curve I). The activity of the corresponding acid-autoclaved extracts (Curve II) between pH 3 and 7 was practically identical with that of the non-hydrolyzed samples, while the biological activity of the alkaline extracts was markedly increased by the acid treatment. This difference in activity was most pronounced with the sodium hydroxide extract, which prior to hydrolysis (point *A*) was practically inactive but exhibited striking activity following autoclaving with acid (point *B*). It is also apparent that the material soluble at pH 3 was not extractable by dilute sodium hydroxide.

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[1] Sufficient 0.2 N sodium hydroxide was added to a solution which was 0.04 M with respect to phosphoric, acetic, and boric acids to give buffers of the desired pH.

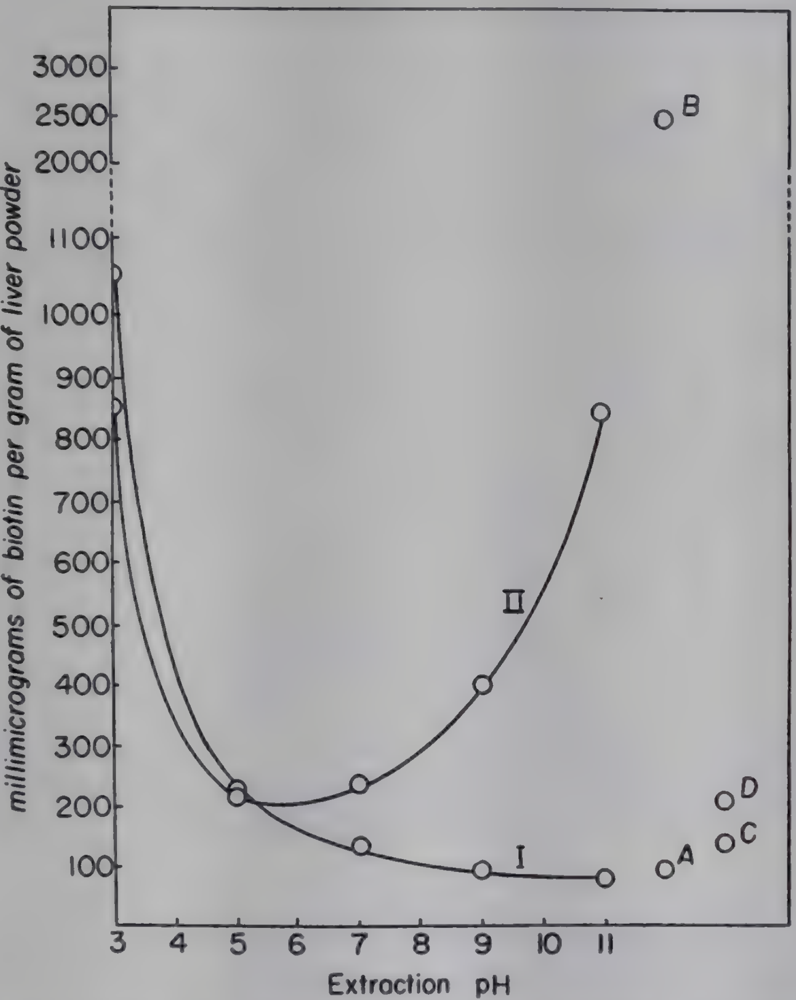

FIG. 1. Biotin activity of extracts obtained from acetone liver powder. Curve I, unhydrolyzed extracts; Curve II, hydrolyzed extracts. *A*, biotin content of NaOH extract before hydrolysis; *B*, biotin content of NaOH extract after hydrolysis; *C*, biotin content of water extract before hydrolysis; *D*, biotin content of water extract after hydrolysis.

These results indicated that two different biotin-containing fractions were obtained from the liver powder, one with the pH 3 buffer and the other with sodium hydroxide. Neither fraction could be extracted with distilled water (points *C* and *D*). Accordingly, experiments were undertaken with the aim of recovering both these materials from the same sample of liver powder by first extracting with universal buffer at pH 3 and then with dilute sodium hydroxide. The results of such an experiment are illustrated in Fig. 2, where it may be seen that both fractions could be recovered by this procedure. Again the pH 3 extract did not show an increase in biological activity following acid hydrolysis, in contrast to the alkali-soluble material which became highly active under these conditions. It is especially noteworthy that 90 to 100 per cent of the biotin present in the liver powder may be recovered in the two ex-

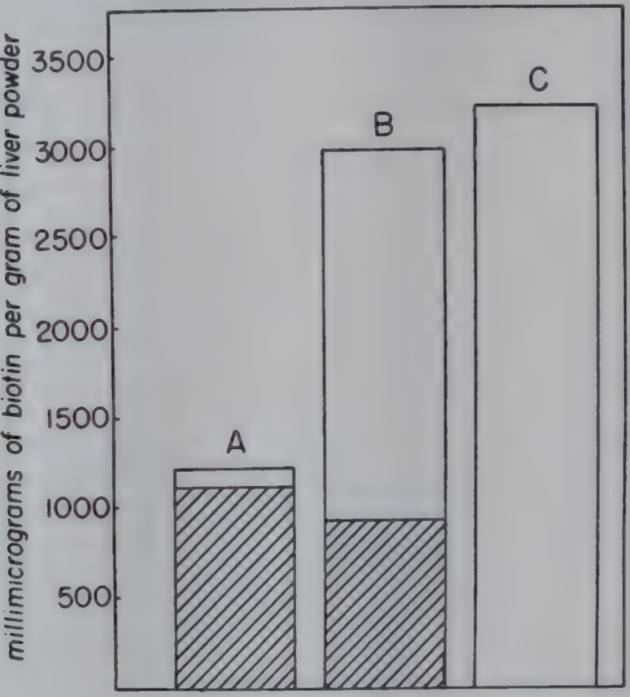

FIG. 2. Recovery of biotin in extracts from liver powder. *A*, biotin content of non-hydrolyzed extract at pH 3 (shaded) and non-hydrolyzed NaOH extract (clear). *B*, biotin content of hydrolyzed extract at pH 3 (shaded) and hydrolyzed NaOH extract (clear). *C*, total biotin content of liver powder.

TABLE I

Behavior of Liver Fractions on Dialysis

The figures represent millimicrograms of biotin.

Extract	Biotin before dialysis		Biotin after dialysis	
	Acid-hydrolyzed	Non-hydrolyzed	Acid-hydrolyzed	Non-hydrolyzed
pH 3	1060	1000	80	90
NaOH	1530	220	1510	70

tracts. Attempts to recover the material soluble at pH 3 from liver powder previously extracted with sodium hydroxide were unsuccessful.

The different nature of the two fractions was further apparent from their behavior on dialysis as shown in Table I. It may be noted that dialysis for 48 hours resulted in an almost complete loss of the biological activity of the pH 3 extract. The alkali-soluble material is virtually non-dialyzable; its low activity prior to acid hydrolysis seems to be due to contamination with a dialyzable biotin-active compound of low molecular weight.

The lack of activity of the alkali-soluble material may be explained in two ways. Either the biotin is combined with a high molecular substance (protein) and is thus not available to the microorganism, or the extracts contain acid-labile inhibitory compounds which suppress its growth-promoting activity. The latter possibility was ruled out by recovery experiments summarized in Table II, in which known amounts of biotin were added to the non-hydrolyzed sodium hydroxide extracts. The excellent recoveries of biotin in the presence of either the dialyzed or non-dialyzed extract demonstrate the absence of inhibitory substances.

TABLE II

Recovery of Biotin Added to Sodium Hydroxide Extract

Biotin content of extract	Biotin added	Recovery of added biotin	
mμgm.	*mμgm.*	*mμgm.*	*per cent*
0.04*	0.02	0.02	100
0.04*	0.05	0.04	80
0.04*	0.15	0.14	93
0.04*	0.20	0.23	115
0.05†	0.02	0.03	150
0.05†	0.05	0.05	100
0.05†	0.10	0.12	120

* Non-hydrolyzed sodium hydroxide extract.

† Non-hydrolyzed dialyzed sodium hydroxide extract.

The existing nomenclature of the so called "bound forms" of biotin is at present in a confused state. Bowden and Peterson (2), for example, applied the term "soluble bound forms" of biotin to substances obtained by digestion of liver with pepsin or partial hydrolysis with sulfuric acid. Our findings which demonstrate that the biotin activity present in beef liver is extractable under mild conditions not involving such hydrolytic treatments suggest the need for a different nomenclature. In accordance with the accepted term flavoprotein, we propose that the term *biotoprotein* be applied to *biotin-containing proteins* as they occur in tissues. The biotin activity may be available to microorganisms either before or after hydrolysis. We assume that our sodium hydroxide-soluble material represents such a biotoprotein. The low molecular weight biotin-containing portion of this biotoprotein may well be identical with the substance described by Bowden and Peterson (2). Experiments to test this hypothesis are presently under way. The similarity in the bacterial spectrum of biocytin and Bowden and Peterson's material indicates the possible identity of these two compounds.

The nature of the material soluble at pH 3 is less clearly definable, as it contains biotin activity in a rather loose combination which is cleaved by simple dialysis against distilled water. The solubility characteristics make it rather unlikely that this material is biotin.

Recently it has been suggested that biotin may in some manner function in the fixation of carbon dioxide (8–12). Several enzymes involved in the decarboxylation of β-keto acids have been obtained in purified form; however, in no instance could the enzymatic activity be correlated with the biotin content (10, 13, 14). Thus it becomes evident that the elucidation of the mode of action of biotin will depend in the final analysis on the isolation in pure form of biotin-containing enzymes and the identification of their specific catalytic activity. The work herein described represents a first step towards this goal.

SUMMARY

The biotin activity present in acetone-dried beef liver was almost quantitatively extracted by successive treatments with universal buffer at pH 3 and dilute sodium hydroxide. Two distinct fractions containing biotin were thus obtained. The substance soluble at pH 3 possessed biotin activity which was not increased by acid hydrolysis; on the other hand, the sodium hydroxide-soluble material exhibited activity only after treatment with acid. In contrast to the fraction soluble at pH 3 which lost its biotin activity on dialysis, the biological activity of the alkali-soluble substance remained practically unaltered by this treatment. The chemical nature of the alkali-soluble substance was discussed and it was postulated to be a biotin-protein complex. The name biotoprotein was proposed for native biotin-containing proteins occurring in tissues.

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A MODIFICATION OF THE CARBAZOLE REACTION OF HEXURONIC ACIDS FOR THE STUDY OF POLYURONIDES*

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In a previous report a specific color reaction of hexuronic acids with carbazole and sulfuric acid was described (1). In this reaction glucuronic and galacturonic acids, in free as well as in combined form in polyuronides, reacted with almost equal intensity. By decreasing the concentration of the acid and the temperature and time of heating, a modification of the carbazole reaction was obtained in which the intensity of the color depends not only on the structure of individual hexuronic acids but also on specific linkages in polyuronides. The present report deals with this modification. It will be referred to as the reaction at 60° to distinguish it from the first modification, the reaction at 100°.

EXPERIMENTAL

Procedure—5.4 cc. of a mixture of 1 part of water plus 6 parts of concentrated sulfuric acid, c.p., are pipetted into test-tubes of equal inner and outer diameter, which are immersed in ice. The acid is cooled to 0°, 0.4 cc. of a solution containing 0.05 to 0.5 mg. of hexuronic acid per cc. is added, and the mixture is shaken in ice. After the reaction mixture has cooled to 0°, it is held for 3 minutes in tap water at room temperature and immersed immediately in a water bath at 60° for 90 seconds. It is then cooled in tap water, and 0.2 cc. of a 0.1 per cent solution of carbazole (Eastman Kodak preparation, twice recrystallized from benzene) is added and vigorously shaken. A purple color develops slowly and increases in intensity for several days. To obtain as great differences in color intensity as possible with individual hexuronic acids and polyuronides, the intensity of the color should be measured 1 hour after addition of carbazole against a blank containing 0.4 cc. of water instead of the unknown solution. If a Klett photoelectric colorimeter is used as the optical instrument, the maximum absorption is obtained with Filter 54. With the Beckman spectrophotometer, the maximum absorption is found at 527 mμ.

Sensitivity and Specificity of Reaction—The color can be detected with

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solutions containing as little as 0.05 mg. per cc. of galacturonic or 1 mg. per cc. of glucuronic acid. The reading on the Klett colorimeter in this case is about 20. Hexoses, pentoses, methylpentoses, and desoxyribose nucleic acid do not give an appreciable color even in concentrations of 0.5 mg. per cc.

Influence of Proteins—The intensity of the color reaction is influenced by the proteins in the solution. Serum proteins at a concentration of 0.1 per cent depress the intensity of the color by 25 per cent. The effect is not perceptible with 0.02 per cent solutions. Therefore, if the concentration of hexuronic acids is not high enough to allow a dilution sufficient to decrease the protein concentration below 0.02 per cent, it is necessary to determine the percental depressing effect of the protein on the color reaction. This can be done by adding a known amount of galacturonic acid to a certain volume of solution and measuring the increase of color against a standard prepared by adding the same amount of hexuronic acid to a corresponding volume of water.

Intensity of Color Given by Various Hexuronic Acids and Polyuronides in Reaction

The intensity of color given in this procedure by equivalent amounts of hexuronic acids in free and conjugated form shows (Table I) great variations with the nature of the acid and of the polyuronide. Free galacturonic acid yields a color 30 times more intense than that from glucuronic acid. Among the polyuronides, two groups with very different behaviors are conspicuous. To the first group belong the two pneumococcus polysaccharides (types I and III) and pectic acid. Their reaction is negligible. They behave in the same way as the β-mentholglucuronide. It is obvious that the glycosidic linkage by which the hexuronic acid is bound in these compounds depresses the color they would show as free hexuronic acids.

The second group is represented by the three mucopolysaccharides containing an amino sugar: the hyaluronic and chondroitinsulfuric acids from cartilage, heparin, and alginic acid. In spite of the decrease in color due to glycosidic linkages, these polyuronides show a considerable intensity of color which, in the case of hyaluronic acid, is about 20 times stronger than that from an equivalent amount of the free glucuronic acid[1] (measured with Filter 54 in the Klett photoelectric colorimeter).

Extinction Coefficient in Color Reaction as Characteristic of Polyuronides

The great variations in the reactivity of different polyuronides in this reaction permit their characterization by the extinction coefficient, ϵ_{527}.

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[1] The ratio between color intensities of glucuronic acid and polyglucuronides cannot be determined very accurately because of the low values obtained for glucuronic acid. This ratio depends also somewhat on the room temperature.

After the total amount of hexuronic acid in a preparation or unknown solution containing polyuronides is determined (by one of the modifications of the Lefèvre-Tollens method), the intensity of color as determined in the present method will enable one in many cases to detect the nature of the polyuronide. To this end, the density of color formed from our preparation is compared with that given by a standard solution of glucuronic or galacturonic acid containing as much total hexuronic acid as the unknown.

Table I

Color Intensity Given by Various Hexuronic Acids and Polyuronides in Reaction at 60°

Readings 1 hour after addition of carbazole.

Experiment No.	Substance	Concentration hexuronic acid	Galvanometer reading*
		mg. per cent	
I	Glucuronic acid	83.3	18
	Chondroitinsulfuric acid†	85	62
	Chondridin	100	83
II	Glucuronic acid	83.3	13
	Chondroitinsulfuric acid	85	53
	Hyaluronic acid	168	250
III	Pneumococcus type III polysaccharide	90	3
IV	Glucuronic acid	80	14
	β-Menthoglucuronide	80	0
	Hyaluronic acid from corpus vitreum	80	189
	Hyaluronic acid from umbilical cord	80	210
	Chondroitinsulfuric acid	80	52
	Galacturonic acid	25	84
	Pectic acid	25	8
V	Galacturonic acid	12.5	46
	Pectic acid	12.5	0
	β-Mentholglucuronide	100	0
	Hyaluronic acid from corpus vitreum	40	70
VI	Glucuronic acid	50	3
	Pneumococcus type I polysaccharide	100	7
	Chondroitinsulfuric acid	40	16
VII	Glucuronic acid	100	10
	Hyaluronic " from umbilical cord	100	214
	Galacturonic acid	25	75
	Pectic acid	25	5

* Readings made with a Klett photoelectric colorimeter.

† All preparations of chondroitinsulfuric acid used in this investigation were prepared from cartilage. I am greatly indebted to Dr. Karl Meyer for the preparations of hyaluronic and chondroitinsulfuric acids and to Dr. Michael Heidelberger for the bacterial polysaccharides.

The relative density of the two solutions will indicate the nature of the polyuronide.

The determination of the total hexuronic acid, according to Lefèvre and Tollens, can be replaced by the determination of the intensity of color given by the unknown solution in the carbazole reaction at 100° previously described (1). To this end, the relative density of the unknown is determined in the two procedures by comparison with standard solutions containing 0.05 to 0.1 mg. of galacturonic acid per cc. The relative density obtained in the reaction, which involves heating at 100°, of equimolar solutions of all hexuronic acids and polyuronides so far investigated is very nearly the same. The ratio of the relative densities given by the two methods designated $D_{\text{R}60^\circ}/D_{\text{R}100^\circ}$, therefore, is as characteristic for many polyuronides (hyaluronic, chondroitinsulfuric, and pectic acids) as the extinction coefficient in the reaction at 60° (see Table II).

TABLE II

Ratio of Relative Densities of Reactions at 60 and 100°

$D_{\text{R}60^\circ}/D_{\text{R}100^\circ}$ for various polyuronides against a standard of galacturonic acid of 25 mg. per cent.

Experiment No.	Temperature	Substance	Concentration hexuronic acid	Galvanometer reading*	$D_{\text{R}60^\circ}$	$D_{\text{R}100^\circ}$	$\frac{D_{\text{R}60^\circ}}{D_{\text{R}100^\circ}}$
	°C.		mg. per cent				
I	24	Hyaluronic acid from corpus vitreum	100	214	0.72	1.42	0.51
		Pectic acid	25	5	0.06	1	0.06
		Galacturonic acid	25	75			
II	28	Hyaluronic acid from corpus vitreum	80	189	0.76	1.42	0.53
		Chondroitinsulfuric acid from cartilage	80	52	0.19	1.14	0.17
		Galacturonic acid	25	84			
III	30	Pneumococcus type I polysaccharide	12.5	0	0	1.15	
		Alginic acid	40	61	0.51	0.25	2
		Galacturonic acid	25	74			
IV	28	Heparin crystalline Ba salt	20	74	1.21	2.05	0.59
		Galacturonic acid	25	76			

* Readings made with a Klett photoelectric colorimeter.

DISCUSSION

The new modification of the carbazole reaction promises to be useful for three different lines of investigation.

Preliminary experiments have shown that the intensity of the color formed following the reaction at 60° is proportional to the concentration of galacturonic acid in the range between 0.05 to 1.0 mg. per cc. and of hyaluronic acid and heparin between 0.05 and 0.25 mg. per cc. Therefore, it should be possible to determine these compounds in the presence of an excess of free glucuronic acid, β-glucuronides (in urine), and other polyuronides.

The 60° and 100° reactions can be used in combination for testing the purity of preparations of polyuronides, at least as far as contamination by other polyuronides is concerned. To this end, the ratio $D_{R60°}/D_{R100°}$ is compared with the ratio of a reference preparation of known purity. Any impurity in the form of a polyuronide should, in general, give rise to a difference in this ratio in the two preparations.

Finally, the close relation between the intensity of color and certain structural peculiarities of polyuronides should make it possible to differentiate between and to characterize certain closely related mucopolysaccharides isolated from different animal tissues. Meyer *et al.* (2) have reported that preparations of chondroitinsulfuric and mucoitinsulfuric acids from various organs show considerable differences as far as certain physicochemical properties (viscosity, rotation) and sensitivity towards enzymes are concerned. These authors considered the possibility that these differences in behavior indicate differences in structure. These physicochemical properties and the susceptibility to enzyme activity, however, cannot be regarded as unequivocal criteria of the chemical individuality of compounds of the character of mucopolysaccharides. The degree of polymerization of the latter, as well as impurities, may affect those properties. On the other hand, experiments[2] have shown that the intensity of color given by hyaluronic acid in the reaction at 60° does not depend on the degree of polymerization. Normal sulfuric acid, which at 20° strongly affects the viscosity of solutions of hyaluronic acid, does not affect its chromogenic properties at all. Obviously this reaction depends on a specific structure of a low molecular unit of the polysaccharide.

The fact that certain polyuronides like hyaluronic acid yield much more color following the reaction conducted at 60° than does free glucuronic acid can be easily understood if one considers the general mechanism of the breakdown of sugars in strong acids which precedes the color reactions with the organic chromogenic reagent. As a rule, several intermediary products more or less related to, or derived from, furfural and its homologues will arise. Of these, in general, only one will combine with the developer to form the colored product ("active intermediary"). The ratio between the amount of the active and the other intermediaries will often depend on the presence of free hydroxyl groups in certain positions in the sugar molecule. If one of these groups is bound by a glycosidic linkage, a shift of this ratio towards the active intermediary may result. If the active product is present in much lower concentration than the inactive (as was shown to be the case in the diphenylamine reaction of desoxypentose (3)), such a shift may result in a considerable increase in the intensity of the color reaction.

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[2] Unpublished data.

SUMMARY

A modification of the carbazole reaction for hexuronic acids is described, which permits a differentiation between galacturonic and glucuronic acids and between various types of polyuronides.

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A STUDY OF THE BASIS OF SELECTIVITY OF ACTION OF ANTIMETABOLITES WITH ANALOGUES OF PIMELIC ACID

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Since selectivity of action of a drug is one of the prime factors which determines its therapeutic usefulness, any attempt to approach chemotherapy of both infectious and non-infectious diseases by means other than those dependent on chance must necessarily require some insight into the reasons why pharmacological agents show the ability to affect some organisms or tissues, and not to touch others. In all probability, a variety of bases for this selectivity exists. This paper is concerned with the investigation of one of them.

The selectivity of sulfanilamide and its related drugs in being able to harm bacteria but not to destroy higher animals has been postulated (1) to arise from the fact that the animals cannot synthesize folic acid, while the pathogenic microorganisms can do so. These drugs are antimetabolites of *p*-aminobenzoic acid and apparently owe their effects to competition with this metabolite in the synthesis of folic acid (2). This idea about the reason for their selective action arose from the observation of Lampen and Jones (3) that those bacteria which require folic acid preformed are not harmed by the drugs. Because most higher animals require dietary folic acid, they lack the metabolic system which sulfonamide drugs inhibit, and therefore are not vitally affected by them, while pathogenic bacteria which do form folic acid may be susceptible to the inhibition.

In order to test this postulate further, it was applied to another vitamin in an effort to make a selective agent. An antimetabolite related to the precursor of the vitamin was therefore prepared and tested on a series of living things. Biotin was selected for this trial because pimelic acid has been shown to be a specific precursor from which, by a series of reactions, the vitamin is formed (4). Furthermore, many animal species seem to require a dietary source of biotin, while some pathogenic microorganisms, *e.g.* the tubercle bacillus, do not. If the hypothesis is correct, one would expect that an effective analogue of pimelic acid would harm those organisms which can synthesize their own biotin, while it should not affect those animals and microorganisms which do not. Furthermore, the harmful effects of such an analogue should be overcome competitively by pimelic acid and non-competitively by small amounts of biotin.

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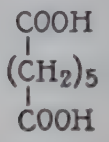
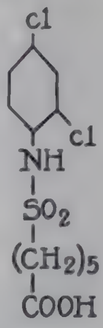
* With the technical assistance of N. Smith and J. Cowperthwaite.

Analogues of pimelic acid which will do these things in the species thus far examined have been made. The most active one was the ε-dichlorosulfanilide of caproic acid. Its structure and that of pimelic acid are shown in the diagram. The simpler analogue in which a plain sulfonamido group replaced the dichlorosulfanilido radical was effective, but with the bacteria tested the inhibition of growth which it caused disappeared as the time of incubation was increased. However, the monosulfonic acid analogue of pimelic acid, namely ε-sulfonocaproic acid, was not harmful to any organism tested, and therefore was not an antimetabolite of pimelic acid.



$$\begin{array}{c} COOH \\ | \\ (CH_2)_5 \\ | \\ COOH \end{array}$$

Pimelic acid



ε-[2,4-Dichloro-sulfanilido]
-caproic acid

The successful prediction of these results by use of the hypothesis lends some support to it as an explanation of one of the bases of selectivity of action of antimetabolites. It does not constitute proof of its validity but does make it seem advisable to explore it further.

EXPERIMENTAL

Sodium and Barium Salts of ε-Sulfonoethyl Caproate—To a hot solution of 22 gm. of ε-bromoethyl caproate (5) in 50 cc. of ethanol was added slowly with stirring a solution of 23 gm. of Na_2SO_3 in 40 cc. of water. The mixture was heated on the steam bath under a reflux and stirred vigorously for 1 hour.[1] It was then concentrated under reduced pressure to dryness and the residue was extracted with 400 cc. of hot ethanol. After filtration the solution was concentrated under reduced pressure to a sirup which was extracted with 400 cc. of ether. This removed 7.7 gm. of unchanged bromo ester. The ether-insoluble matter was dissolved in ethanol and precipitated by the addition of 10 volumes of ether. This operation was repeated twice; yield 12 gm. of white powder. This material was mainly the sodium salt of ϵ-sulfonoethyl caproate but contained some of the disodium salt of the corresponding caproic acid. The separation of these two sodium salts proved difficult, but the two acids could be parted completely as barium salts. To do this 500 mg. of the mixed sodium salts were dissolved in 25 cc. of ethanol and a solution of 500 mg. of barium iodide in 10 cc. of ethanol was added. Shiny crystals of the barium salt of ϵ-sulfonoethyl caproate soon began to form, and these were recrystallized by adding 4 volumes of ethanol to a 10 per cent solution of them in water. For the preparation of the sulfonamido and sulfanilido compounds, the mixed sodium salts were satisfactory.

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[1] This time of reaction was chosen arbitrarily. Systematic investigation might reveal conditions which would minimize hydrolysis of the ester and thus increase the yield.

$C_{16}H_{30}O_{10}S_2Ba$.	Calculated.	C 33.0, H 5.2, Ba 23.6
	Found.	" 32.5, " 5.2, " 23.8

ϵ-*Sulfonamidocaproic Acid*—2.5 gm. of the sodium salt of ϵ-sulfonoethyl caproate (mixed with small amounts of the disodium salt of the corresponding caproic acid) were ground in a mortar with 5 gm. of PCl_5. When the reaction had subsided, the mixture was extracted with 50 cc. of dry benzene and the filtered solution was concentrated under reduced pressure to dryness. Dry benzene was added and the evaporation was repeated to remove HCl and $POCl_3$. The acid chloride crystallized but was used without further purification. 100 cc. of concentrated aqueous ammonium hydroxide were added and the solution which resulted was kept at room temperature for 2 days, and then concentrated under reduced pressure to dryness. The diamide of ϵ-sulfonocaproic acid was separated by extraction of the residue with absolute ethanol and fractional precipitation with ethyl acetate. It did not crystallize, but rather formed as an amorphous powder. This was therefore dissolved in 25 cc. of 6 N HCl and the solution was refluxed for 4 hours. The reaction mixture was concentrated under reduced pressure to dryness, dissolved in water, and from this solution the sulfonamide was extracted with ethyl acetate. Evaporation of the extract left a crystalline substance which was recrystallized from ethyl acetate solution by adding benzene. Yield 660 mg.; m.p. 102–104°.

$C_6H_{13}O_4NS$. Calculated, N 7.2; found, N 7.3

ϵ-*(2,4-Dichlorosulfanilido)-caproic Acid*—The acid chloride from 2.5 gm. of the crude sodium salt of ϵ-sulfonoethyl caproate was prepared as described in the previous section and was dissolved in 10 cc. of dry benzene. This solution was added to 4 gm. of 2,4-dichloroaniline dissolved in 20 cc. of benzene. Since reaction seemed slow the solvent was removed under reduced pressure and the residue was heated at 120° for 30 minutes. The product was dissolved in ethyl acetate, and this solution was washed several times with 1 N HCl and then with water. The crude sulfanilido ester which was obtained by concentration of the ethyl acetate was not purified further, but rather was converted to the free acid by refluxing it for 2 hours in 32 cc. of 1 N alcoholic NaOH. The ethanol was removed under reduced pressure, the residue was dissolved in water, and the solution was extracted with ethyl acetate. Acidification of the aqueous phase gave 1.5 gm. of the desired acid which was recrystallized by adding benzene to an ethyl acetate solution. It melted at 115–118°.

$C_{12}H_{15}O_4NSCl_2$. Calculated, C 42.4, H 4.41; found, C 42.4, H 4.65

Method of Testing with Microorganisms—To test a pimelic acid analogue for ability to inhibit growth of microorganisms, the substance was added to a medium free of biotin and of pimelic acid. Graded amounts ranging from 1 mg. per cc. of culture medium down to 1 γ were so tested with each species and with each compound. For those forms which needed biotin for growth, an amount of this vitamin equal to about half that which gave maximal response was included in the basal medium. For all except *Acetobacter*, *Saccharomyces*, and the tubercle bacillus, the basal medium described by Landy and Dicken (6) was used. In order to minimize any possible interference with the inhibitory action of the analogues due to excess amino acids, the amount of casein hydrolysate in the basal medium was reduced to one-fourth that recommended. With this quantity of amino acids, excellent growth was still obtained. The basal medium therefore contained glucose, acid hydrolysate of casein, tryptophan, cystine, asparagine, sodium acetate, potassium phosphate, sodium chloride, salts of iron, manganese, and magnesium, adenine, guanine, xanthine, uracil, thiamine, riboflavin, pantothenic acid, pyridoxine, pyridoxamine, nicotinic acid, folic acid, and *p*-aminobenzoic acid. *Acetobacter suboxydans* was tested in the medium described for it by Landy and Dicken (7), and for *Saccharomyces cerevisiae* the synthetic medium used by Woolley and White (8) was employed. The experiments with *Mycobacterium tuberculosis* were conducted by Dr. R. J. Dubos in the basal medium which he has developed for this organism (9).

When one of the analogues was found to inhibit the growth of a given species, additional tests were made to determine (*a*) whether pimelic acid would overcome the inhibition, (*b*) whether biotin would overcome the inhibition, and (*c*) whether these antagonisms were competitive. To do this, a series of graded dilutions of the analogue was tested in the presence of no added metabolite, with 1 γ per cc. of pimelic acid, with 3 γ per cc. of pimelic acid, and with 0.0005 γ and with 0.005 γ of biotin. For each amount of either metabolite the quantity of the analogue which gave half maximal inhibition of growth was determined from a plot of the dose-response curve. Inspection of these values then readily showed whether antagonism existed, and if so, what its nature was. A competitive antagonism would demand a 3-fold increase in analogue when 3 times as much metabolite was present, in order to achieve similar effect. If it were non-competitive, the same amount of analogue would suffice at all levels of the metabolite. Except where noted otherwise, all periods of incubation were 2 days at 37°. Extent of growth was measured quantitatively with an Evelyn colorimeter. *A. suboxydans*, *S. cerevisiae*, *Lactobacillus arabinosus*, and *Leuconostoc mesenteroides* were grown at 30° instead of 37°. The organisms were all cultured in 25 × 100 mm. test-tubes containing 10 cc. of medium except in the case of *A. suboxydans*, *S. cerevisiae*, and *Bacillus tenuis*, in which cases 50 cc. Erlenmeyer flasks were used. This manner of cultivation was important, as will be shown below for *B. tenuis*.

Microorganisms Used—The selection of organisms for testing depended on their nutritional needs for biotin. While biotin-requiring species are common, those with no dependence on this vitamin are rare. Some forms which previously had been reported not to require biotin were found to do so provided that the inoculum was thoroughly washed and diluted. *B. tenuis*, a close relative of *Bacillus subtilis*, was found very useful in this work, because although it would grow without added biotin, addition of this vitamin enhanced multiplication. Furthermore, pimelic acid would replace biotin in this respect. *A. suboxydans* likewise grew somewhat without biotin and was stimulated when it was present, but pimelic acid was not able to replace it.

B. tenuis and *Proteus* 4 were kindly supplied by Dr. R. J. Dubos. *A. suboxydans* was from the American Type Culture Collection. A biotin-requiring mutant of *Escherichia coli* was kindly supplied by Dr. B. Davis and Dr. W. Maas. All other species were those previously in use in this laboratory.

Stimulation of Growth of B. tenuis by Pimelic Acid or by Biotin—When *B. tenuis* was inoculated into the biotin-free basal medium just described, growth was scant and slow to begin, unless either biotin or pimelic acid was present. This effect was regularly noted when 10 cc. of medium were employed in 25 × 100 mm. test-tubes. However, when the cultivation was in 50 cc. Erlenmeyer flasks, growth in the unsupplemented basal medium was faster, and the stimulation by biotin and by pimelic acid was not evident. In the trials in test-tubes, growth was at first diffuse throughout the medium. In the unsupplemented tubes, this diffuse character remained. In the supplemented tubes growth began as a uniform turbidity but a pellicle soon developed on the surface. These pellicles complicated the determination of the amount of growth because grinding was required to bring them into uniform suspension. In the cultures in flasks, no trouble was experienced from pellicle formation. Data to illustrate the stimulation of growth by pimelic acid and by biotin are shown in Table I. These readings were taken after 2 days of incubation. In order to eliminate variations in the results which arose from failure to obtain uniform suspensions of the organism for inoculation, the inoculum was prepared as follows. The cells from 1 cc. of a 1 day-old culture in a 1 per cent tryptose-glucose medium were collected and washed twice. They were then resuspended uniformly by mixing with 1 cc. of sterile saline and grinding briefly in a TenBroeck glass grinder. The suspension so obtained was free of clumps and was diluted 1:100 and 1 drop of this dilution was used per flask or per tube in the tests.

TABLE I

Growth Response to Biotin and to Pimelic Acid of B. tenuis Cultured in Test-Tubes and in Flasks

Addition to basal medium	Turbidity*	
	In tubes	In flasks
None	84	65
Biotin, 0.001 γ per cc.	63	61
" 0.0003 " " "	64	63
" 0.0001 " " "	88	67
Pimelic acid, 10 γ per cc.	75	
" " 3 " " "	71	64
" " 1 " " "	81	62

* Expressed as per cent of incident light transmitted by the culture when compared to uninoculated medium.

From the data in Table I it is clear that pimelic acid can replace biotin, but that much more of the former than of the latter was required. The same situation has been shown with the diphtheria bacillus (4).

Because of the more rapid growth obtainable in flasks, all subsequent trials with *B. tenuis* were conducted in flasks, even though the stimulation by biotin and pimelic acid was not then demonstrable.

Inhibition of Growth of B. tenuis by ε-(2,4-Dichlorosulfanilido)-caproic Acid—The data in Table II will show that small amounts of this sulfanilide inhibited the growth of *B. tenuis* under the conditions just described.

Antagonism between ε-(2,4-Dichlorosulfanilido)-caproic Acid and Pimelic Acid—The amounts of the analogue needed to cause half maximal inhibition of the growth of *B. tenuis* were determined in the presence of no added pimelic acid, and with 1 γ of it per cc. and with 3 times this quantity. The results are shown in Table III where it can be seen that pimelic acid was able to overcome the action of the analogue competitively (*cf.* also Table II). If the amount of analogue needed when no pimelic acid was added is subtracted from the quantities necessary in the presence of 1 γ and of 3 γ of the metabolite, approximately 3 times as much additional analogue was required with 3 γ of pimelic acid as with 1 γ.

Antagonism between ε-(2,4-Dichlorosulfanilido)-caproic Acid and Biotin—When slightly more biotin than that which would cause stimulation of growth of *B. tenuis* cultured in test-tubes (see Table I) was added to the basal medium, then quantities of the analogue which previously had completely suppressed growth no longer caused any inhibition. Under these conditions of biotin addition no effect of the analogue was noted until about 2 mg. per cc. were present. With such large concentrations of the analogue (*i.e.* above 2 mg. per cc.) neither biotin nor pimelic acid would overcome the inhibition of growth. At this high concentration the analogue probably shows a toxicity unrelated to its antimetabolite effects. Sulfonamide drugs in high concentration likewise have been found to behave similarly in that *p*-aminobenzoic acid will not nullify their action (2).

TABLE II

Inhibition of Growth of B. tenuis Cultured in Flasks by ε-(2,4-Dichlorosulfanilido)-caproic Acid and Its Reversal by Pimelic Acid or Biotin

Analogue	Biotin	Pimelic acid	Turbidity*
γ per cc.	γ per cc.	γ per cc.	
0	0	0	70
20	0	0	60
50	0	0	82
100	0	0	98
100	0.0005	0	66
100	0	3	70

* See foot-note to Table I.

TABLE III

Amounts of ε-(2,4-Dichlorosulfanilido)-caproic Acid Which Caused Half Maximal Inhibition of Growth of B. tenuis in Presence of Various Quantities of Pimelic Acid

Pimelic acid added	Analogue needed for half maximal inhibition
γ per cc.	γ per cc.
0	58
1	85
3	150

The antagonism between the sulfanilide and biotin would thus appear to be strictly non-competitive. Once the biotin which the bacillus would ordinarily elaborate from pimelic acid has been added from external sources, the inhibition caused by interference with this process is completely removed.

Correlation of Toxic Properties of ε-(2,4-Dichlorosulfanilido)-caproic Acid with Nutritional Requirements of Various Species for Biotin—A variety of microbial species was tested for inhibition of growth by the sulfanilido analogue in the manner described in a preceding section. The ability of each species to grow in the basal medium free of biotin likewise was determined. The results are shown in Table IV. For species of which inhibition of growth occurred the action of the analogue was found to be overcome by biotin or by pimelic acid. Except for *E. coli*, the species which required biotin were not inhibited, and those which did not, were.

TABLE IV

Correlation of Toxicity of ε-(2,4-Dichlorosulfanilido)-caproic Acid with Nutritional Needs for Biotin

Organism	Analogue needed for half maximal inhibition	Nutritional requirement for biotin
	γ *per cc.*	
Bacillus tenuis	58*	Not required, but stimulatory under certain conditions
Acetobacter suboxydans	350	Not required, but somewhat stimulatory
Mycobacterium tuberculosis H37Rv	20†	Not required
Escherichia coli	No effect at 1000	" "
" " *biotinless*	" " " 1000	Required
Proteus strain 4	" " " 1000	"
Staphylococcus aureus	" " " 300	"
Lactobacillus casei	" " " 500	"
" *arabinosus*	" " " 500	"
Leuconostoc mesenteroides	" " " 500	"
Hemolytic streptococcus H69D	" " " 500	"
Saccharomyces cerevisiae	" " " 1000	"

* When cultured in test-tubes, a value of 20 was uniformly found.

† Incubation time 4 days.

A fault in the argument seems to appear in these tests, because the presence of biotin in the medium is needed in order to test the biotin-requiring species for any effect of the analogue. We have just seen that biotin will nullify the effects of the analogue on the non-biotin-requiring species. In an effort to resolve this difficulty, the amount of biotin used in the tests with the biotin-requiring forms was limited to less than the concentration necessary for maximal growth response. It was hoped that in this way any possible inhibition caused by the analogue might be unmasked. Some slight support for this hope was found with *B. tenuis* cultured in test-tubes in the presence of suboptimal amounts of biotin. Growth could be inhibited by the analogue but never completely. Instead, it was usually reduced to that obtainable with the suboptimal amount of biotin alone. Nevertheless, no adequate means of dealing with this fault in the experimental set-up has been found.

Effect of ε-(2,4-Dichlorosulfanilido)-caproic Acid on Biotin Synthesis by E. coli—A second fault in the argument appears from the finding that *E. coli* was not inhibited in growth by the analogue. Since this organism does not require biotin, one would expect it to be susceptible. In an effort to study this problem, the effect of the analogue on the synthesis of biotin by *E. coli* was examined. The bacteria were grown in the biotin-free basal medium for 2 days. Various amounts of the analogue ranging from 0 to 500 γ per cc. were added at the start of this period of growth. At the end, the cultures were heated at 100° for 5 minutes, centrifuged, and the supernatant solutions were analyzed for biotin according to the method of Eakin *et al.* (10). In the unsupplemented basal medium 0.008 γ of biotin per cc. of culture was found, but no diminution in this amount was detected in the cultures grown in the presence of the greatest concentration of the analogue. Therefore, it seemed plain that the analogue had not inhibited the synthesis of biotin in this organism, just as it had not inhibited growth.

A plausible explanation of the failure of the analogue to inhibit the growth of *E. coli* might be that the organism forms sufficient pimelic acid to antagonize completely any practicable amount of the analogue. In the case of some sulfanilamide-resistant bacteria, the production of large amounts of *p*-aminobenzoic acid seems to account for their resistance to the drug (11). *E. coli* might owe its resistance towards the pimelic acid analogue to a similar ability to form sufficient pimelic acid. The biological assay of pimelic acid is complicated by the fact that biotin which might be present in the sample is far more potent in promoting growth of the organisms that require pimelic acid than is pimelic acid itself. An attempt was made to overcome this difficulty by destruction of the biotin. To this end a filtrate from a heated culture of *E. coli* grown in the biotin-free basal medium for 2 days was acidified to pH 2 and extracted five times with ethyl acetate. The material which was extracted was dissolved in water and treated with hydrogen peroxide to oxidize the biotin which it contained. The reaction mixture was then freed of peroxide with manganese dioxide, acidified, and extracted with ethyl acetate five times. The extracted material was evaporated and assayed for pimelic acid by making use of the stimulation of growth of *B. tenuis* which this acid produces when the organism is grown in test-tubes.[2] A very considerable stimulation was produced. However, the assay of pimelic acid as carried out with *B. tenuis* cannot be considered as conclusive because of uncertainties about its specificity. Therefore, the failure to explain adequately why the analogue does not inhibit *E. coli* must remain a serious fault in the argument about the basis of selectivity of action of this compound. Similar failures of the hypothesis as applied to the sulfonamide drugs could be enumerated, because some sulfanilamide-resistant streptococci are known which still do not require folic acid.

Toxicity of ε-(2,4-Dichlorosulfanilido)-caproic Acid for Mice—Since mice are believed to rely on an alimentary source of biotin, one might expect that the analogue of pimelic acid would not be harmful to these animals. To test this, young adult mice of the Rockefeller Institute strain were injected intraperitoneally with neutral solutions of the compound. Twelve mice so treated with single doses of 10 mg. of the analogue showed no toxic manifestations either immediately or after maintenance for 3 weeks on a stock diet.

Inhibition of Growth of B. tenuis by ε-Sulfonamidocaproic Acid and Its Dependence on Time of Incubation—When *B. tenuis* was grown in flasks for 20 hours, a marked inhibition was caused by this sulfonamide analogue of pimelic acid. Inhibition was complete with 100 γ of the analogue per cc. and half maximal effect was found with 20 γ per cc. Biotin or pimelic acid overcame these actions. However, when incubation was continued, growth began in the flasks containing the analogue, and after 2 days no retardation was found even with 500 γ per cc.

Stimulation of Growth by Subinhibitory Amounts of ε-(2,4-Dichlorosulfanilido)-caproic Acid—Concentrations of the analogue which were too small to cause inhibition of growth of *B. tenuis* produced stimulation. This effect was considerably greater when pimelic acid or biotin was added to the basal medium than in the absence of these addenda. The data of Table II will show the magnitude of the stimulation in the absence of these vitamins. Thus, like many other antimetabolites (12), this analogue of pimelic acid caused stimulation of growth when present in subinhibitory concentrations.

SUMMARY

ε-(2,4-Dichlorosulfanilido)-caproic acid, an analogue of pimelic acid in which the dichlorosulfanilido group replaced one of the carboxyls of the metabolite, has been shown to inhibit the growth of several bacteria which

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[2] It was also assayed for biotin and found to contain less than 1 per cent of that present in the original culture.

do not require biotin as a growth factor and not to affect those which do require biotin. One species, *E. coli*, was found which was insusceptible to the action of the analogue and yet did not require biotin. It thus deviated from the simple relationship. These facts were offered in support of a hypothesis to explain one of the bases of selectivity of action of antimetabolites. This postulate has been promulgated previously for the sulfonamide drugs derived from *p*-aminobenzoic acid. According to it a selective agent can be produced by forming a structurally similar inhibitor of a *precursor* of a chosen metabolite. Such an analogue will then affect those species which can synthesize this metabolite, but will not harm those which cannot, and which rely on dietary sources of the chosen metabolite. The limitations of the experimental findings in relation to this hypothesis have been indicated.

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THE FORMATION OF 2,3-DIPHOSPHOGLYCERATE IN RABBIT ERYTHROCYTES: THE EXISTENCE OF A DIPHOSPHOGLYCERATE MUTASE

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The red blood cells of most mammalian species are distinguished by the presence of 2,3-diphosphoglyceric acid, which accounts for about half of their total acid-soluble P (1). The compound is stable in contrast to the labile 1,3-phosphoglyceric acid (2), which is considered an obligatory intermediate in the glycolytic cycle of all tissues. From studies with radioactive P it would appear that 2,3-diphosphoglycerate has a rapid turnover comparable to that of other phosphate esters of the red cells (3), an indication that it lies either in the main path of their phosphate cycle or exists in free equilibrium with an obligatory intermediate. The mechanism of its formation is unknown. Similar uncertainty obtains with regard to the steps in its decomposition. In intact cells at a plasma pH below 7.35, breakdown occurs which is linked with maintenance of adenosine triphosphate (ATP) (4). In the unfortified hemolysate the compound is almost inert (5), but is decomposed readily when adenylic acid is added (6).

The present study is concerned with the mechanism of the formation of 2,3-phosphoglycerate (2,3-P-GA).

Methods

Most of the experiments were performed with hemolysates of rabbit erythrocytes, prepared by the addition of 2 volumes of distilled water to 1 volume of cells twice washed in the cold with 0.9 per cent NaCl. Hemolysates of beef cells were prepared in the same manner. Phosphate determinations were performed by the method of Fiske and Subbarow (7). Acid hydrolysis of the acid-soluble P was carried out for 15 or 150 minutes in N sulfuric acid in a boiling water bath. Total phosphoglycerate was determined by the naphthoresorcinol method (8) adapted to the photoelectric colorimeter, and 3-phosphoglycerate by polarimetry (9).

Results

Formation of Stable P in Rabbit Hemolysates—The formation of difficultly hydrolyzable P was studied in a system consisting of a hemolysate of rabbit erythrocytes containing iodoacetate and fluoride, incubated in the presence of ATP, 3-phosphoglycerate (3-P-GA), or both. The iodoacetate served to inhibit formation of phosphoglycerate by way of the phosphoglyceraldehyde dehydrogenase system, and the fluoride, decomposition of phosphoglycerate through enolase activity. In the amounts added, both inhibitors were highly effective. Table I presents the data of a representative experiment. It may be seen that some liberation of inorganic P occurred when ATP was added. The amount was greatest in Tube A, which contained both ATP and 3-P-GA. Disappearance of hydrolyzable P in significant amounts occurred only when both compounds were added.

Table I

Disappearance of Added Substrates from Rabbit Hemolysate

The incubation mixtures contained, per ml. of final volume, 0.5 ml. of a freshly prepared 1:3 water hemolysate of rabbit cells, 25 μM of fluoride, and 20 μM of iodoacetate. Where indicated in the table, 3 μM of 3-P-GA and approximately 2 μM of ATP were added. All additions were made in the form of sodium salts. The samples were incubated for 4 hours at 37°. The values are expressed as micromoles per 0.5 ml. of red cells.

Experimental conditions		Phosphate analysis						3-P-GA	
		Inorganic		15 min. hydrolysis		150 min. hydrolysis			
		Total	Δ*	Total	Δ*	Total	Δ*	Total	Δ*
Tube O + ATP + 3-P-GA	Initial	1.5		14.7		18.4		10.0	
" A + " + "	Incubated	5.0	+3.6	10.0	−4.7	13.7	−4.7	5.4	−4.6
" B + "	"	4.4	+2.9	13.4	−1.3	17.5	−0.9	0.0	0.0
" C + 3-P-GA	"	1.4	−0.2	2.4	−0.1	3.4	0.0	10.0	0.0
" D	"	1.6		2.5		3.4		0.0	

* Differences of Tubes A and B *versus* O, and of Tube C *versus* D.

Independent determination of 3-P-GA by the polarimetric method, listed in the last column of Table I, indicates the disappearance of an amount of 3-P-GA equivalent to that of labile P transferred to the stable form.

These data would indicate the formation of 2,3-diphosphoglycerate from 3-P-GA and ATP, in consonance with an over-all reaction:

$$\text{(1)} \qquad \text{3-P-GA} + \text{ATP} = \text{2,3-P-GA} + \text{ADP}$$

Although a one-step transphosphorylation according to Reaction 1 above is a possibility, it would appear more likely that the reaction proceeds in two steps as follows:

$$\text{(2)} \quad \text{3-P-GA} + \text{ATP} \rightleftarrows \text{1,3-P-GA} + \text{ADP} \text{ (phosphokinase of Bücher (10))}$$

$$\text{(2, } a\text{)} \qquad \text{1,3-P-GA} \rightarrow \text{3-P-GA} + \text{inorganic P}$$

(3) $$\text{1,3-P-GA} \underset{?}{\rightleftarrows} \text{2,3-P-GA}$$ (diphosphoglycerate mutase)

Reaction 2, because of the lability of 1,3-P-GA, should lead to an increase of inorganic P without change in the sum of inorganic and hydrolyzable P. The occurrence of Reaction 3, catalyzed by the mutase in question, would be indicated by the transfer of P from the hydrolyzable to the stable form. The occurrence of the Bücher reaction in rabbit hemolysates is suggested by the accelerated liberation of inorganic P when both ATP and 3-P-GA are present.

In Fig. 1 is presented the time curve of stable P formation, and in Figs. 2 and 3 the relation of the synthesis to the concentration of ATP and 3-P-GA. In Fig. 4 the pH-activity curve is portrayed, together with one presenting the sum of inorganic and stable P formed, both derived from ATP. It may be seen that formation of stable P has a pH optimum between 6.9 and 7.5, with rapid loss of activity on either side. The total amount of P transferred from ATP to either inorganic or stable form has a similar optimum, but a somewhat less steep fall of activity, particularly on the acid side.

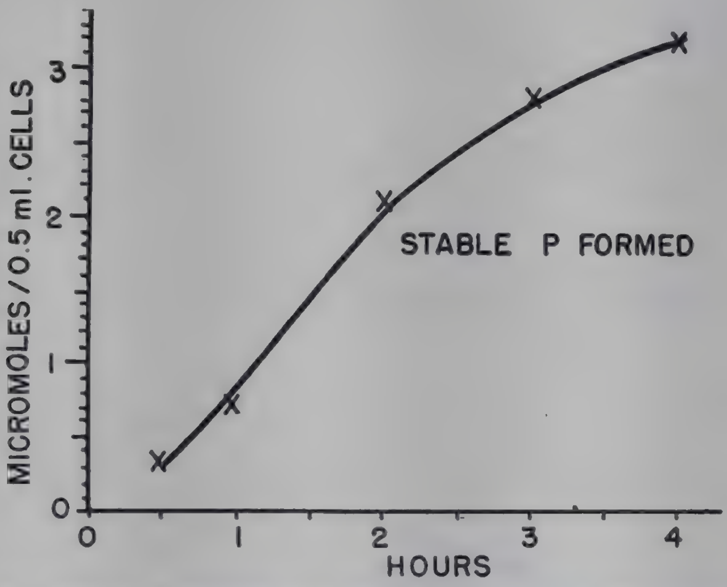

FIG. 1. Time curve of formation of stable P in rabbit hemolysate. The experimental conditions were the same as those described in Table I, except for the variation in incubation time.

In Table II are presented data on the effect of dilution of the hemolysate on the formation of stable P. It may be seen that, while the liberation of inorganic P reached a plateau and showed little change on further dilution, the formation of stable P declined. Such behavior may be expected in a system of stepwise reactions controlled by two or more enzymes. Apparently the rate of 2,3-P-GA formation decreased greatly with falling concentrations of 1,3-P-GA and fell below the rate of the spontaneous decomposition of the labile compound.

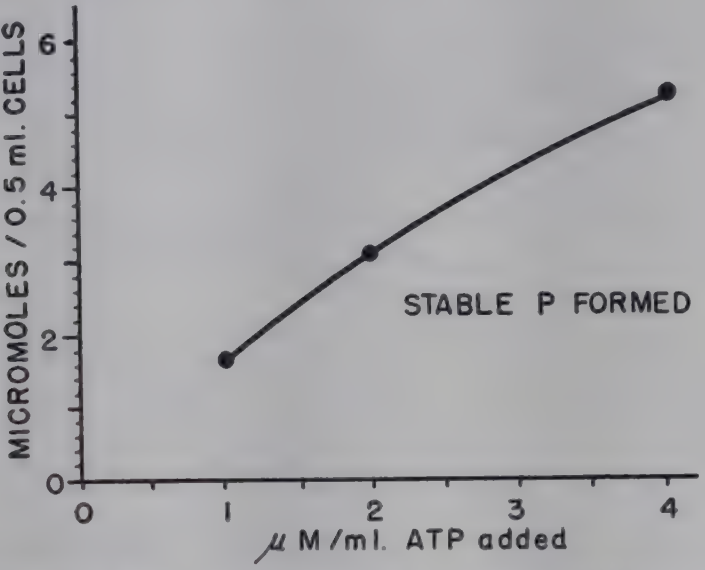

FIG. 2. The effect of ATP. The conditions were the same as previously described, except for the variation in the amount of ATP added.

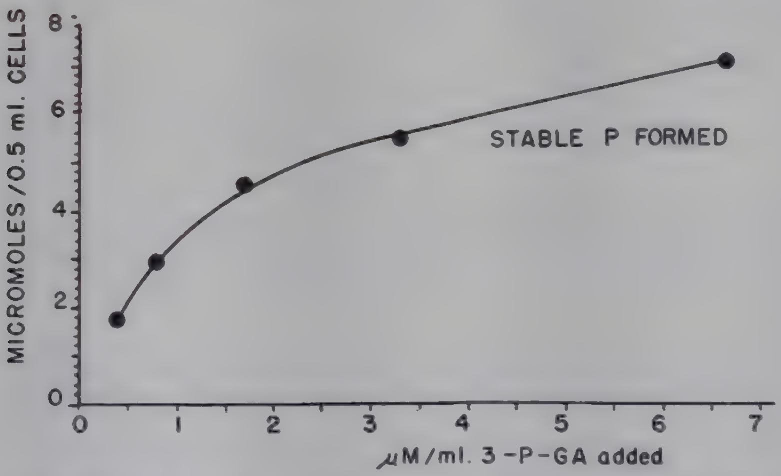

FIG. 3. The effect of 3-P-GA. The conditions were the same as before, except for the variation in 3-P-GA.

Beef Cells and Muscle—It appeared of interest to study beef cells which do not contain diphosphoglycerate. Such cells, like those of other tissues, are known to form 3-P-GA when incubated with fluoride and pyruvate, rather than diphosphoglycerate, which is formed by rabbit erythrocytes which contain diphosphoglycerate (11). Presumably they lack the enzyme necessary for the formation of 2,3-P-GA. In Table III is shown a representative experiment on beef hemolysate. It may be seen that, as expected, the beef cells are inactive in the formation of stable P. The moderate activity in liberating inorganic P, particularly in the complete system containing both ATP and 3-P-GA, is an indication of the presence of the Bücher enzyme. In Table IV are shown data on experiments with acetone powder of a water extract of rat muscle. It may be seen that no

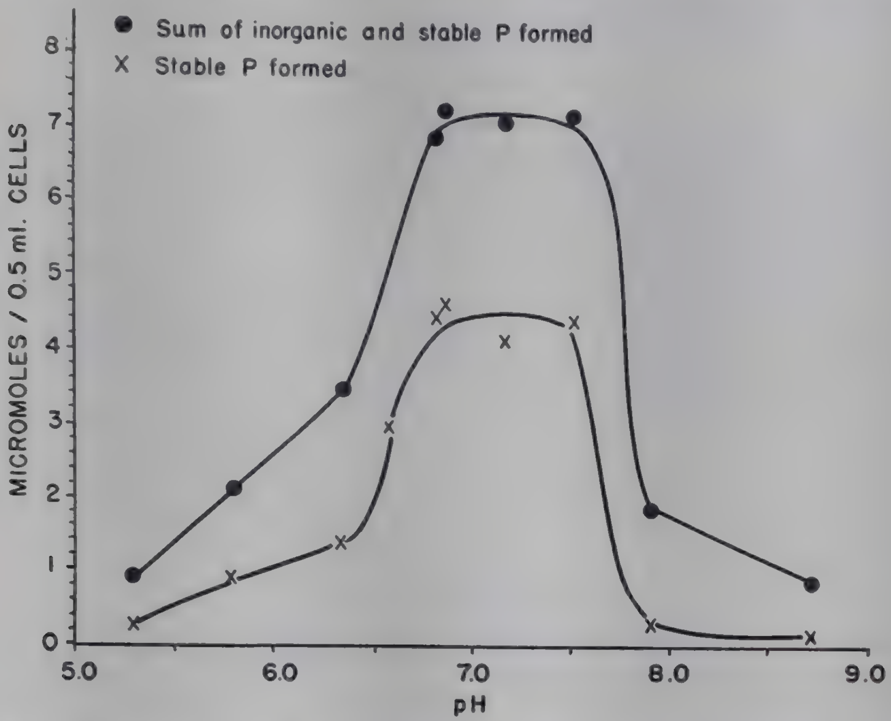

FIG. 4. pH-activity curve of rabbit hemolysate. The experimental conditions were the same as those described in Table I. Different pH values were produced by additions of HCl or NaOH of appropriate strength, and the exact values determined with a glass electrode.

TABLE II

Effect of Dilution of Hemolysate

The conditions were the same as those described for Experiment A in Table I, except for the variation in the amount of red blood cells added, as indicated in the table. The values are expressed in micromoles.

Red cells per ml. mixture	Inorganic P formed		Stable P formed	
	Actual	Per 0.5 ml. cell	Actual	Per 0.5 ml. cell
ml.				
0.167	1.7	5.1	1.4	4.3
0.083	1.3	8.1	0.6	3.3
0.042	0.7	8	0.1	1
0.021	0.4	9		−1

formation of stable P occurred; rather, an increase in the total hydrolyzable P took place. A comparison of the 150 minute P values of Tubes 4 and 6 shows that the increase was primarily due to a breakdown of 3-P-GA, presumably owing to an incomplete inhibition of enolase. The presence of an active Bücher enzyme is indicated by the high inorganic P value in Tube 4, which contained both ATP and 3-P-GA.

TABLE III

Beef Cell Experiment

The experiment was set up in the same fashion as that described in Table I, except for the use of a 1:3 hemolysate of beef cells instead of rabbit cells. The samples were incubated for 4 hours at 37°. The values are expressed in micromoles per 0.5 ml. of red blood cells.

Experimental conditions		Inorganic P		150 min. hydrolysis	
		Total	Δ	Total	Δ
Tube O + ATP + 3-P-GA	Initial	2.2		17.8	
" A + " + "	Incubated	4.3	+2.1	17.8	0.0
" B + "	"	3.5	+1.3	18.1	+0.3

TABLE IV

Muscle Experiment

The experiment was performed as described in Table I, except that the mixtures contained approximately 4 μM per ml. of ATP. 0.1 ml. of a 10 per cent suspension of acetone powder of water extract of rat muscle, adjusted to pH 6.8 with $NaHCO_3$, was added per ml. of final mixture. The values are expressed as micromoles per 0.1 ml. of muscle suspension.

Experimental conditions			Inorganic P		150 min. hydrolysis	
			Total	Δ*	Total	Δ*
Tube 1 + muscle alone		Initial	3.1		3.6	
" 2 + " "		Incubated	3.5	+0.4	3.8	+0.2
" 3 + muscle + ATP	+ 3-P-GA	Initial	4.7		12.7	
" 4 + muscle + ATP	"	Incubated	7.3	+2.6	14.0	+1.3
" 5 + muscle + ATP		"	5.0	+0.3	12.5	−0.2
" 6 + muscle	+ 3-P-GA	"	4.4	+0.9	4.9	+1.1

* Differences of Tube 2 *versus* Tubes 1 and 4, Tube 5 *versus* 3, and Tube 6 *versus* 2.

Effect of Muscle on Erythrocytes—It appeared of interest to study the effect of the muscle preparation on the formation of stable P in hemolysates. An accelerating effect of the muscle, by itself inactive but containing a potent Bücher enzyme, would strongly support the hypothesis of a two-step reaction. In Table V are presented two such experiments, one at 37° and the other at 25°, both of which show a significant increase in the formation of stable P when the muscle powder was added. The effect of varying concentrations of muscle powder on the red blood cell system is presented in Table VI. It may be seen that, although the inorganic P formation increased with increasing amounts of muscle, a plateau of stable P formation was reached. In other experiments, even inhibition occurred with the largest additions of muscle powder.

TABLE V

Accelerating Effect of Muscle on Formation of Stable P in Rabbit Hemolysate

The conditions were the same as those described previously, except that the mixtures contained approximately 4 μM per ml. of ATP. 0.1 ml. of a 10 per cent suspension of acetone powder of water extract of rat muscle, adjusted to pH 6.9 with $NaHCO_3$, was added per ml. of mixture. In one experiment unneutralized muscle suspension, pH 6.2, was used. The incubation times and temperatures are given in the table. The values are expressed as micromoles per 0.5 ml. of red cells.

Experimental conditions		Tube	Inorganic P formed	Stable P formed
hrs.	*°C.*			
4	25	With muscle	7.2	2.5
		Without muscle	3.4	1.7
2	37	With muscle, pH 6.2	5.5	3.7
		" " " 6.9	6.3	2.3
		Without muscle, pH 6.8	2.6	1.8

TABLE VI

Effect of Increasing Amounts of Muscle on Stable P Formation

The conditions were the same as in Table V. Varying amounts of a 10 per cent aqueous suspension of acetone powder of muscle extract, adjusted to pH 6.5, were added. The samples were incubated for 4 hours at 37°. The values are expressed as micromoles per 0.5 ml. of red cells.

Muscle suspension per ml. final volume	Inorganic P formed	Stable P formed
ml.		
0.0	3.8	4.3
0.05	5.7	4.5
0.10	7.7	6.9
0.15	8.7	7.0
0.20	9.7	7.0
0.10 (No 3-P-GA)	6.7	0.0

Fractionation of Rabbit Hemolysate with Ammonium Sulfate—More decisive evidence concerning the two-step mechanism of 2,3-P-GA formation could be obtained by fractional precipitation of the hemolysate with ammonium sulfate. The results of an illustrative experiment are shown in Table VII. It may be seen that the enzyme tended to distribute itse in the fractions precipitated by 0.50 to 0.75 saturation with ammoniu sulfate. A single fractionation reduced greatly the amount of stable formed without addition of muscle enzyme. On repeated fractionatio of Fractions II and III, 0.50 and 0.66 saturated with ammonium sulfate stable P formation no longer was demonstrable without addition of musc

TABLE VII

Ammonium Sulfate Fractionation of Rabbit Hemolysate

About 20 ml. of rabbit red blood cells, washed twice with cold 0.9 per cent NaCl solution, were hemolyzed by freezing and by addition of 1 volume of distilled water. To the hemolysate which had a pH of 6.9, 0.33 volume of saturated ammonium sulfate solution was added to produce 0.25 saturation. The precipitate containing most of the stroma was separated by centrifugation, and to the clear red supernatant fluid were added, in succession, amounts of saturated ammonium sulfate solution sufficient to produce the saturations listed in the table. The bulk of the hemoglobin was contained in the 0.75 fraction. The precipitates were taken up in small volumes of distilled water and dialyzed overnight against running distilled water. The precipitates which appeared in the dialyzing bags were removed since they proved inactive. The combined fractions 0.50 and 0.66, which contained most of the activity and little hemoglobin, were brought to a pH of 5.8 by addition of 0.1 volume of 0.2 M acetate buffer and refractionated with ammonium sulfate. The various precipitates were redissolved in distilled water and dialyzed. They were practically free of hemoglobin.

Activity tests were performed in a manner similar to that described in Table V with and without addition of acetone powder of muscle. The inorganic P values for the tests with muscle are omitted from the table since they offer no information beyond indicating activity of the muscle powder. The values are expressed as micromoles of P per total fraction. The initial total activity of the red cells was about 180 μM of stable P formed in 4 hours.

Fraction No.	pH	Ammonium sulfate saturation	Inorganic P formed	Stable P formed	
				Without muscle	With muscle
I	6.9	0.25	57	9	55
II		0.50	29	23	55
III		0.66	20	24	62
IV		0.75	10	3	3
II + III					
a	5.8	0.33	7	0	0
b		0.50	13	0	35
c		0.66	8	0	29
d		0.80	6	0	16

powder. Liberation of inorganic P decreased to a small per cent of the original value.

DISCUSSION

The data obtained leave little doubt that formation of 2,3-diphosphoglycerate was observed under circumstances which establish ATP and 3-P-GA as precursors. The evidence concerning a two-step reaction, the first catalyzed by the Bücher enzyme leading to production of 1,3-P-GA which then is transformed to 2,3-P-GA by a mutase, consists in the retarding effect of dilution on the synthesis, the accelerating effect of muscle powder, and in the fractionation of the hemolysate with ammonium sulfate until formation of stable P no longer occurred in the absence of added muscle powder. The enzyme, which may be called "diphosphoglycerate mutase," presumably is restricted to erythrocytes containing 2,3-P-GA. From the data of Bücher (10) one may conclude that in the present experiments the concentration of 1,3-P-GA must have been very low, much lower than the preexistent 2,3-P-GA concentration in the hemolysates. Occurrence of synthesis under such conditions suggests that the equilibrium of the mutase reaction must be far to the side of the stable compound. Aside from this observed fact, one may raise the question on thermodynamic grounds as to whether the reaction can be reversible at all, in view of the great differences in energy content between the carboxyl phosphate of 1,3-P-GA on the one hand and that of the secondary alcohol phosphate of 2,3-P-GA on the other. An energy difference of 13,000 calories between the two types of groups may be estimated. Such an objection would overlook the fact that a similar system, namely that between 2-P-GA and phosphopyruvate, is freely reversible, with little change in energy, and has an equilibrium constant of 2 to 3 (12). This apparent paradox is explained by the fact that the energy change at the phosphate bond is compensated by an equivalent change in the phosphate-free portion of the compound. Thus free glyceric acid is richer in energy than either pyruvic acid or the acid anhydride (13). In view of these considerations the occurrence of a back-reaction cannot be ruled out entirely.[1]

Work concerning this question is in progress.

SUMMARY

In the presence of adenosine triphosphate and 3-phosphoglycerate disappearance of hydrolyzable P is observed in rabbit hemolysates and interpreted as formation of 2,3-P-GA.

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[1] On the other hand, a biomolecular reaction mechanism, involving 3-P-GA as a coenzyme analogous to that of the phosphoglyceric mutase reaction (14) (suggested by Professor C. F. Cori in a personal communication), would lead to irreversible 2,3-P-GA formation.

A two-step reaction is assumed with formation of 1,3-P-GA, catalyzed by the phosphokinase of Bücher, preceding its transformation to 2,3-P-GA. The enzyme responsible for the second reaction, "diphosphoglycerate mutase," is absent from beef erythrocytes and muscle and is thought to be restricted in occurrence to red cells containing 2,3-P-GA. A partial purification of the enzyme has been achieved.

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CONVERSION OF LACTATE TO LIVER GLYCOGEN IN THE INTACT RAT, STUDIED WITH ISOTOPIC LACTATE*

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The distribution of isotope in liver glycogen deposited following the feeding of a labeled compound may be used as an indicator *in vivo* of intermediary reactions linking the fed compound and glycogen. When acetate or butyrate labeled with isotopic carbon is administered together with non-isotopic glucose to a fasted rat, the resulting liver glycogen contains isotope (3, 4). If the distribution of the isotope in the glycogen is determined by hydrolysis and degradation of the resulting glucose, a number of characteristic patterns are observed (4). These distribution patterns are consistent with the reactions of the tricarboxylic acid cycle and of glycolysis as the main paths for the transfer of carbon from these non-carbohydrate sources to glycogen.

In the present experiments the metabolism of lactic acid has been studied by this technique. Three types of labeled lactate,[1] $CH_3 \cdot C^{13}HOH \cdot COONa$, $C^{13}H_3 \cdot C^{13}HOH \cdot COONa$, and $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ have been fed to fasted rats, and the resulting liver glycogen hydrolyzed to glucose and degraded. The distribution of isotope has been found to be in harmony with predictions based on the schemes of glycolysis and the tricarboxylic acid cycle. The results, when interpreted in terms of these schemes, indicate that the major fraction of the administered lactate carbon which was converted to glycogen traversed reactions of the tricarboxylic acid cycle prior to this conversion.

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[1] Racemic mixtures of the D and L forms were fed in each case. Calculations involving the amount of isotope administered have been based on the isotope content of the total lactate fed, although it might have been more appropriate to consider only the isotope contained in the natural form. The possible metabolic rôle of the unnatural isomer in determining the results of the present experiments has otherwise been left out of consideration.

Methods

The lactate labeled with C^{13} in the α and β positions was synthesized according to the method of Cramer and Kistiakowsky (5) with some modifications. The C^{13}-α-labeled lactate was prepared from $CH_3 \cdot C^{13}OOH$ by esterification with butyl alcohol, and hydrogenation of the ester to the alcohols with copper-barium-chromium oxide catalyst, yielding $CH_3 \cdot C^{13}H_2OH$ and $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2OH$. The mixture of alcohols was converted to the corresponding iodides and fractionated. The ethyl iodide was converted by the Grignard reaction to propionic acid, $CH_3 \cdot C^{13}H_2 \cdot COOH$, which was isolated as the sodium salt. The final steps may be represented as follows:

$$CH_3 \cdot C^{13}H_2 \cdot COONa \xrightarrow{P\ +\ Br} CH_3 \cdot C^{13}HBr \cdot COOH \xrightarrow{Na_2CO_3} CH_3 \cdot C^{13}HOH \cdot COONa$$

The $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ was prepared in a generally similar manner. $C^{14}H_3C^{13}OOH$ was made from $C^{14}H_3I$ and $KC^{13}N$. Conversion of the ethyl iodide to propionate was accomplished by nitrile formation and hydrolysis. The propionate was boiled with benzoyl chloride and the resulting propionyl chloride was removed by distillation. Bromination and hydrolysis yielded the isotopic lactate. Purity of the different acetic and propionic acids was checked by partition coefficient (6), which agreed with theory within experimental error. In all cases, the lactic acid was removed from the hydrolysis mixture by continuous ether extraction. Total acid, by titration, agreed with lactate determined colorimetrically (7).

The general plan of the experiments and the methods employed were similar to those described previously (4, 8). Male white rats, after a 24 hour fast, were given the isotopic lactate as the sodium salt by stomach tube, together with non-isotopic glucose to enhance glycogen deposition.[2] In several experiments, the respiratory CO_2 was collected. 3 hours after giving the lactate, the livers were removed under amytal anesthesia, and the glycogen isolated and hydrolyzed to glucose. When necessary, the glucose from more than one liver was pooled.

The glucose was converted to lactate by fermentation with *Lactobacillus*

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[2] In some experiments preliminary to the present work, C^{13}-α,β-labeled lactate was administered in larger doses and without glucose. Not only were the glycogen yields much smaller, but the level of isotope in the glycogen was not much greater than that found in the present experiments. Comparable results were obtained by Vennesland *et al.* (9) who also administered α,β-labeled lactate without glucose. If its use is not contraindicated by other considerations, the administration of glucose thus permits economy of isotopic compound and makes for conveniently large glycogen yields with little decrease in isotope concentration in the glycogen.

casei, and the lactic acid was carried through a stepwise degradation to yield the carboxyl and α- and β-carbons of the lactate as separate fractions (4, 8). These fractions correspond to positions 3 and 4, 2 and 5, and 1 and 6 of the original glucose, respectively. In the experiments with $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ (Experiments 6 to 9) the fraction consisting of carbons 1 and 6, obtained as iodoform, was purified by sublimation before conversion to CO_2 in an effort to minimize dilution with adventitious, non-isotopic carbon, known to occur at this step (4). In addition, in Experiments 6 to 9 part of the glucose was converted to the methyl glucoside and degraded by an alternative procedure (4, 6). This degradation yields three separate fractions containing carbon 3, carbons 1, 2, 4, and 5, and carbon 6 of the glucose, respectively. The carbon 3 fraction is contaminated with small amounts of carbon from other positions. The various fractions from both types of degradation, after conversion to CO_2, were analyzed for their C^{13} content in the mass spectrometer, as was the respiratory CO_2. In Experiments 6 to 9 duplicate aliquots of all samples were precipitated as $BaCO_3$, and radioactivity determined with an end window Geiger-Müller tube. The method of sample preparation was very similar to that of Henriques *et al.* (10). The radioactivity measurement on the duplicate samples of the glycogen fractions agreed within ±2 per cent. Duplicates on the respiratory CO_2 samples, which contained much less material, agreed within ±5 per cent.

TABLE I

*Degradation of Synthetic Isotopic Lactate**

Type of lactate	Type of isotope	Isotope concentration† in degradation fractions		
		Carboxyl carbon	α-Carbon	β-Carbon
$CH_3 \cdot C^{13}HOH \cdot COONa$	C^{13}	0.00	0.58	0.00
$C^{13}H_3 \cdot C^{13}HOH \cdot COONa$	"	0.01	1.14	1.16
$C^{14}H_3 \cdot C^{13}HOH \cdot COONa$	"	0.00	1.34	0.00
"	C^{14}	0.00	25	2390

* The isotopic compounds were diluted with non-isotopic lactate before degradation.

† In this and subsequent tables, the C^{13} values are given in atom per cent excess, and the C^{14} values in counts per minute per mg. of carbon.

As a check on the synthesis and degradation procedures, the three types of synthetic lactate, after appropriate dilution with non-isotopic lactic acid, were degraded in the same manner as the biological material. The results, presented in Table I, show that the methods of synthesis and degradation are mutually confirmatory. A trace of C^{14} appears in the α-carbon fraction from the $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$.

RESULTS AND DISCUSSION

The general experimental data are summarized in Table II.

In Table III are presented the results of the glycogen degradations. It will be noted that for all types of lactate the isotope occurs predominantly in positions 1, 2, 5, and 6 of the glucose, and to a much lesser extent in carbons 3 and 4. In previous experiments on CO_2 fixation with labeled bicarbonate, positions 3 and 4 contained only about one-sixth the concentration of isotope found in the respiratory CO_2 (8), whereas in all the present studies for which data on respiratory CO_2 are available (Table IV) the relative concentration runs appreciably higher, being about equal to the respiratory CO_2 in Experiment 5. Although the two types of experiment are not strictly comparable, since the labeled CO_2 is being produced metabolically in the present studies, it seems very unlikely that the level of isotope appearing in carbons 3 and 4 in the lactate experiments can be accounted for by CO_2 fixation alone.

TABLE II

General Experimental Data on Rats

Experiment No.	Body weight after fast	Type of lactate administered	Amount of lactate administered per 100 gm. body weight	Amount of glucose administered per 100 gm. body weight	Liver weight	Liver glycogen (as glucose)
	gm.		mM	mM	gm.	mM
1	185	$CH_3 \cdot C^{13}HOH \cdot COONa$	0.85	3.00	6.73	1.18
2	190	"	1.57	1.47	5.49	1.15
3	210	"	1.42	1.33	6.53	
4	200	$C^{13}H_3 \cdot C^{13}HOH \cdot COONa$	1.60	1.40	6.65	0.96
5	180	"	1.78	1.56	5.73	0.83
6	225	$C^{14}H_3 \cdot C^{13}HOH \cdot COONa$	1.96	2.17	7.30	1.33
7	190	"	1.89	2.10	5.00	0.80
8	205	"	1.85	2.06	5.50	0.87
9	205	"	1.81	2.01	6.00	

In the experiments with lactate containing C^{13} only in the α position (Experiments 1 to 3 and 6 to 9), carbons 2,5 of the glucose are seen to contain about one-third more C^{13} than carbons 1,6. When the β-carbon is labeled with C^{14} (Experiments 6 to 9), the relative distribution of isotope between these positions is reversed, although the disparity between carbons 1,6 and 2,5 is not as great as that noted for the C^{13} of the α-carbon.

TABLE III

Distribution of Isotope in Liver Glycogen after Administration of Labeled Lactate to Rats*

Experiment No.	Type of lactate administered	Type of isotope	Isotope concentration in labeled carbon of administered lactate	Isotope concentration in degradation fractions of glucose			Isotope concentration in degradation fractions of methyl glucoside			Per cent of administered isotope recovered in liver glycogen
				Carbon atoms of glucose			Carbon atoms of glucose			
				3,4	2,5	1,6	3	1,2, 4,5	6	
1	$CH_3\cdot C^{13}HOH\cdot COONa$	C^{13}	4.64	0.01	0.10	0.07				5.91
2–3	"	"	4.64	0.05	0.27	0.18				4.23
4	$C^{13}H_3\cdot C^{13}HOH\cdot COONa$	"	5.06	0.15	0.60	0.59				8.1
5	"	"	5.06	0.18	0.94	0.89				10.8
6†	$C^{14}H_3\cdot C^{13}HOH\cdot COONa$	"	6.50	0.07	0.42	0.30				7.36
		C^{14}	11,600	204	679	826				8.93
6–9	"	C^{13}	6.50	0.10	0.43	0.30	0.09	0.30	0.29	4.86‡
		C^{14}	11,600	201	681	742	227	577	756	5.54‡

* See foot-note to Table I.

† 1 mM of glucose was degraded. The remaining 0.33 mM was combined with that from Experiments 7 to 9, and the pooled sample divided equally and degraded by both methods.

‡ These calucations are based on the total isotope administered and the total recovered in liver glycogen in Experiments 6 to 9.

TABLE IV

Data on Respiratory CO_2 of Rats

Experiment No.	Type of lactate administered	CO_2 output in mM			Type of isotope	Isotope concentration* in respiratory CO_2			Per cent of administered isotope recovered in respiratory CO_2
		1st hr.	2nd hr.	3rd hr.		1st hr.	2nd hr.	3rd hr.	
2	$CH_3\cdot C^{13}HOH\cdot COONa$	7.82	5.83	6.44	C^{13}	0.10	0.15	0.08	15.7
5	$C^{13}H_3\cdot C^{13}HOH\cdot COONa$	7.19	6.64	6.82	"	0.13	0.17	0.21	10.8
7	$C^{14}H_3\cdot C^{13}HOH\cdot COONa$	6.99	7.23	6.76	"	0.08	0.24	0.28	17.9
					C^{14}	143	351	476	16.2
8	"	6.69	5.82	6.46	C^{13}	0.05	0.13	0.20	9.64
					C^{14}	92	243	318	9.29

* See foot-note to Table I.

The results obtained by the two degradation procedures in Experiments 6 to 9 are seen to be in good agreement. The values for carbon 6 from the degradation of the methyl glucoside agree, within experimental error, with the values for carbons 1,6 from the glucose degradation. The expected concentrations for the fraction containing positions 1, 2, 4, and 5, calculated from the results for the three fractions of the glucose degradation, are 0.31 atom per cent excess and 576 c.p.m. per mg. of carbon for C^{13} and C^{14} respectively, in agreement with experimental values of 0.30 and 577. Agreement between carbons 3,4 and 3 is satisfactory, in view of the low C^{13} values and the fact that the carbon 3 fraction contains a small amount of material from other positions.

With the C^{13}-α,β-labeled lactate, the isotope values for carbons 2,5 and 1,6 in Experiment 4 are equal, within the error of the measurement (±0.01 atom per cent). In Experiment 5, positions 2,5 appear to contain about 5 per cent more C^{13} than positions 1,6. This is due to a procedural artifact. As pointed out previously (4) the degradation procedure as usually employed causes a certain amount of dilution of fraction 1,6 with non-isotopic CO_2. This dilution produces a depression of the isotope values for this fraction, which is most marked when the samples are small and when the isotope values are high. In Experiments 2 to 3, at the relatively low C^{13} value of 0.18 atom per cent excess, and large sample size of 0.84 mM, the dilution effect is masked. In Experiment 5, at a higher C^{13} value and smaller sample size (0.89 atom per cent excess and 0.45 mM, respectively), the dilution effect becomes apparent. These dilution effects have been observed by degradation of synthetic lactate, equally labeled with C^{13} in the α and β positions (4).

The data obtained by the simultaneous use of C^{13} and C^{14} in Experiments 6 to 9 permit fairly accurate comparison of the relative contribution of the α- and β-carbons of the lactate to liver glycogen and respiratory CO_2. Since the C^{13} and C^{14} are administered together in the same compound, and determined in aliquots of the same degradation fractions, the significance of any differences noted will not be influenced by variations between animals or chemical procedures, but will depend solely on the errors involved in the isotopic measurements. The values for the per cent of administered isotope recovered in liver glycogen in these experiments, presented in the last column of Table III, indicate that, although the transformation of α- and β-carbons to glycogen is of the same order of magnitude, a larger contribution has been made by the β-carbon. The observed differences lie beyond the errors of the measurements. (In similar experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$ in which, unlike the present experiments, C^{13} and C^{14} were found to be equally distributed between carbons 2,5 and 1,6 of the glucose, the per cent recovery in the glycogen was identical for both isotopes (11).) In the last column of Table IV (Experiments 7 and 8) it will be noted that the per cent of administered isotope recovered in the respiratory CO_2 is approximately the same for both isotopes, indicating that the α- and β-carbons of lactate have contributed about equally to the respiratory CO_2. It might be expected that the introduction of a relatively larger amount of one isotope in the glycogen would result in the appearance of a relatively smaller amount of this isotope in the respiratory CO_2. The differences between the per cent of C^{13} and C^{14} recovered in the respiratory CO_2, while compatible with this expectation both in direction and magnitude, lie within the error of the isotopic measurements on the respiratory CO_2 samples and cannot be considered as significant.

On the assumption that glycogen is formed by reversal of the glycolytic reactions as indicated in Diagram 1 (Reactions 2 and 1), the main results of the present study indicate that isotope administered in either the α- or β-carbon of lactate is largely, though not completely, randomized between these positions in pyruvate, and that, when isotope is introduced in equal concentration in both the α- and β-carbons of lactate simultaneously, the concentration remains equal in the resulting pyruvate.

The reactions of glycolysis and the tricarboxylic acid cycle, presented in abridged form in Diagram 1,[3] readily account for the main results. On the assumption that lactate enters into metabolism via pyruvate, reversal of the glycolytic reactions will lead to the following types of isotopic glucose, $\text{C—}\overset{*}{\text{C}}\text{—C—C—}\overset{*}{\text{C}}\text{—C}$ or $\overset{*}{\text{C}}\text{—C—C—C—C—}\overset{*}{\text{C}}$, depending on whether the isotope is introduced in the α- or β-carbon of the lactate (the asterisk indicates the location of the isotope). CO_2 fixation and the reversible reductive reactions of the tricarboxylic acid cycle (Reactions 3, 10, 9, and 8) followed by Reaction 2, will produce a glucose with isotope equally distributed in carbons 1, 2, 5, and 6 $(\overset{*=*}{\text{C—C}}\text{—C—C—}\overset{*=*}{\text{C—C}})$, regardless of whether the isotope is introduced in the α or β position of the lactate.

The contribution to the isotopic "pyruvate pool" by decarboxylation of oxalacetate formed via the oxidative reactions of the cycle must also be considered. The oxidative reactions of the tricarboxylic acid cycle (Reactions 5 to 10) will involve initially an isotopic oxalacetate (Reaction 3) as well as an isotopic 2-carbon compound (Reaction 4). In the case of $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$, $COOH \cdot C^{14}H_2 \cdot C^{13}O \cdot COOH$ and $C^{14}H_3 \cdot C^{13}OX$ will be the initial reactants. Assuming that the initial condensation reaction occurs on the carbonyl group of oxalacetate, as usually depicted, the newly

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[3] Stern and Ochoa (12) have recently demonstrated citrate accumulation in an aged pigeon liver preparation, using acetate and oxalacetate together in substrate amounts. The preparation was very low in aconitase activity, pointing to citrate as the initial condensation product in the enzyme system involved. Because of this and other recent developments (13, 14), which emphasize the tentative nature of current schemes of the tricarboxylic acid cycle, the individual tricarboxylic acids have been omitted from Diagram 1.

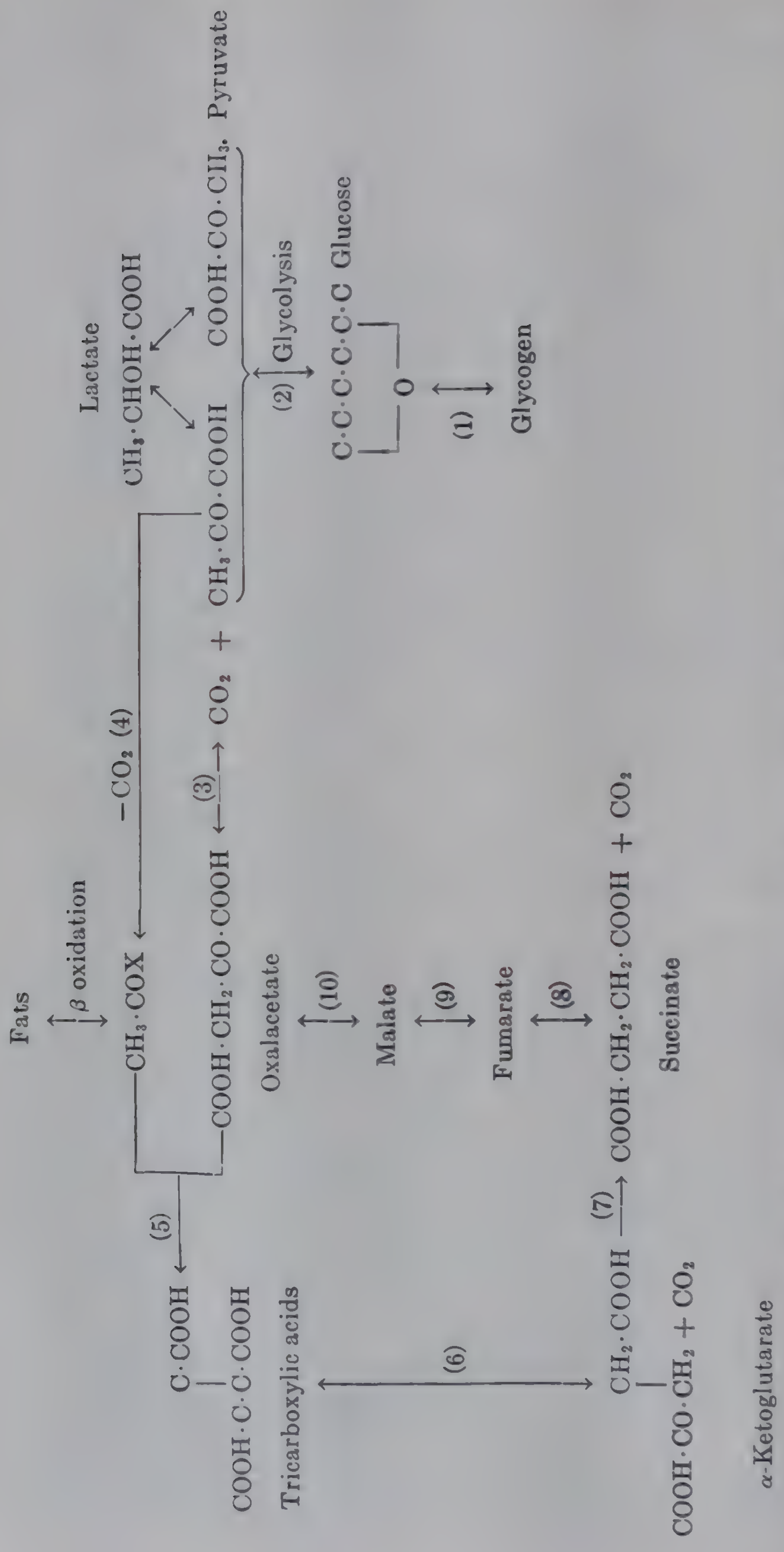
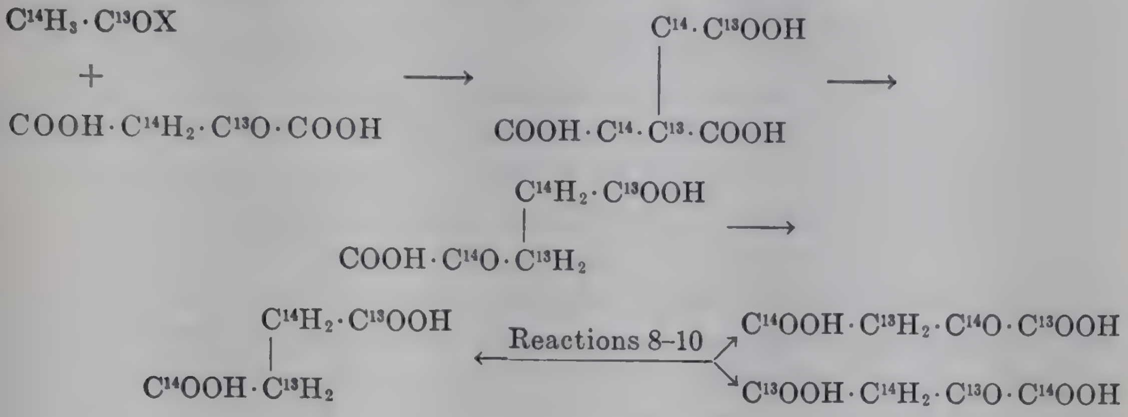

Fats
β oxidation
CH₃·COX
−CO₂ (4)
Lactate
CH₃·CHOH·COOH
(5)
C·COOH
COOH·C·C·COOH
Tricarboxylic acids
COOH·CH₂·CO·COOH
Oxalacetate
(3)
CO₂ + CH₃·CO·COOH
COOH·CO·CH₃. Pyruvate
(2) Glycolysis
C·C·C·C·C·C Glucose
O
(1)
Glycogen
(10)
Malate
(9)
Fumarate
(8)
(6)
CH₂·COOH
COOH·CO·CH₂ + CO₂
(7)
COOH·CH₂·CH₂·COOH + CO₂
Succinate
α-Ketoglutarate

DIAGRAM 1. Abridged tricarboxylic acid cycle and its relationship to glycogen synthesis formed oxalacetate generated via the cycle will contain both isotopes in all positions, since, according to the scheme of Diagram 1,



$$C^{14}H_3\cdot C^{13}OX + COOH\cdot C^{14}H_2\cdot C^{13}O\cdot COOH \longrightarrow \begin{array}{r} C^{14}\cdot C^{13}OOH \\ | \\ COOH\cdot C^{14}\cdot C^{13}\cdot COOH \end{array} \longrightarrow$$

$$\begin{array}{r} C^{14}H_2\cdot C^{13}OOH \\ | \\ COOH\cdot C^{14}O\cdot C^{13}H_2 \end{array} \longrightarrow$$

$$\begin{array}{r} C^{14}H_2\cdot C^{13}OOH \\ | \\ C^{14}OOH\cdot C^{13}H_2 \end{array} \xleftarrow{\text{Reactions 8–10}} \begin{array}{l} \nearrow C^{14}OOH\cdot C^{13}H_2\cdot C^{14}O\cdot C^{13}OOH \\ \searrow C^{13}OOH\cdot C^{14}H_2\cdot C^{13}O\cdot C^{14}OOH \end{array}$$

Any of this oxalacetate going to glycogen via Reactions 3 to 1 will introduce both isotopes into all positions of the hexose chain. C^{13} will be equally distributed among carbons 1, 2, 5, and 6, as will C^{14}. However, the relative abundance of the two isotopes in these positions will depend upon the isotope dilution occurring in the "acetate" and "oxalacetate pools," respectively. For example, heavier labeling in the "acetate pool" should yield a hexose relatively richer in C^{14} than in C^{13}. In addition, under this latter condition, the disparity in isotope concentration between positions 1,6 and 2,5 of the final glycogen, produced by direct conversion of $C^{14}H_3\cdot C^{13}HOH\cdot COONa$ via glycolysis, should be less pronounced in the case of C^{14}, as was actually found.

It will be noted that the oxidative reactions of the tricarboxylic acid cycle provide a path (in addition to CO_2 fixation) for the transfer of isotope from the α- and β-carbons of pyruvate to positions 3,4 of glucose. It may be wondered why the relative concentration of isotope in carbons 3,4 of the glucose is not higher than that actually found in the present study. It should be pointed out that in the presence of a respiratory CO_2 low in isotope, as in the present experiments, the CO_2 fixation reaction may operate to dilute the isotope in carbons 3,4 of the glucose. The contrary would be expected when the respiratory CO_2 is relatively high in isotope, and actually it has been found in similar experiments with $C^{13}H_3\cdot C^{13}OONa$ (4), in which the isotope level in the respiratory CO_2 was several times that obtaining in the present study, that the C^{13} concentration in carbons 3,4 exceeded that in the other positions of the glucose.

Study of the schemes presented in Diagram 1 will also reveal that there is no mechanism for altering complete randomness of distribution of isotope between the α- and β-carbons of pyruvate, once it has been established. As noted (Table III) in the experiments with $C^{13}H_3\cdot C^{13}HOH\cdot COONa$, the resulting liver glycogen contains C^{13} equally distributed between the 1,6 and 2,5 fractions, in accordance with expectations. The reactions of Diagram 1 require, furthermore, that isotope administered in the carboxyl position of lactate should occur exclusively in positions 3 and 4 of the resulting liver glycogen. In experiments with $CH_3 \cdot CHOH \cdot C^{13}OONa$, undertaken in another connection, it has been noted that isotope was present in detectable amounts only in carbons 3 and 4, as expected.

Some idea of the relative amount of isotope that traverses the reactions of the tricarboxylic acid cycle prior to conversion to glycogen via Reactions 2 and 1 may be gained from a consideration of the distribution of isotope in the glycogen in experiments in which α-labeled lactate has been fed. (Similar considerations would apply to the experiments in which β-labeled lactate was administered.) Glucose formed via Reaction 2, before randomization of the isotope has occurred, will exhibit the following isotope distribution pattern, $C{-}\overset{*}{C}{-}C{-}C{-}\overset{*}{C}{-}C$, in the case of α-labeled lactate. As pointed out earlier, CO_2 fixation and the reductive reactions of the tricarboxylic acid cycle (Reactions 3, 10, 9, and 8), followed by Reaction 2, will distribute isotope equally between carbons 1,6 and 2,5 of the resulting glucose ($\overset{*\,=\,*}{C{-}C}{-}C{-}C{-}\overset{*\,=\,*}{C{-}C}$). The CO_2 fixation reaction and the oxidative reactions of the cycle will introduce isotope into carbons 3,4 of the glucose, but will not disturb the equality of distribution between fractions 1,6 and 2,5. The final glycogen in experiments with α-labeled lactate may therefore be considered as having been constituted from essentially two types of isotopic glucose,[4] $C{-}\overset{*}{C}{-}C{-}C{-}\overset{*}{C}{-}C$, and $\overset{*\,=\,*}{C{-}C}{-}\overset{\circ}{C}{-}\overset{\circ}{C}{-}\overset{*\,=\,*}{C{-}C}$. All of the isotope in the second type of glucose, according to the proposed schemes, must have been involved in one way or another in Reactions 3 to 10.

Let us take as an example the distribution of isotope in the liver glycogen in Experiment 6 (Table III). The hexose chain may be pictured thus, $\overset{0.30}{C}{-}\overset{0.42}{C}{-}\overset{0.07}{C}{-}\overset{0.07}{C}{-}\overset{0.42}{C}{-}\overset{0.30}{C}$, in which notation the superscripts are to be taken as representing absolute amounts of C^{13} in each position in the molecule, rather than isotope concentrations.[5] The two types of isotopic glucose from which the final product was constituted may be represented as $C{-}\overset{0.12}{C}{-}C{-}C{-}\overset{0.12}{C}{-}C$ and $\overset{0.30}{C}{-}\overset{0.30}{C}{-}\overset{0.07}{C}{-}\overset{0.07}{C}{-}\overset{0.30}{C}{-}\overset{0.30}{C}$. Thus it appears that the amount of C^{13} that has been involved in the reactions of the tricarboxylic acid cycle prior to deposition in glycogen is about 5 times as great (1.34 to 0.24) as that which has gone directly to glycogen via glycolysis. If the C^{14} data from the same experiment are handled similarly, a ratio of the order of 10:1 is obtained. It should be borne in mind that the differences in isotope content between carbons 1,6 and 2,5, on which these ratios are based, are not very large compared to the errors involved in estimating the isotope concentrations.

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[4] The symbols indicate the relative isotope concentration, $* > \circ$.

[5] For purposes of comparison, the absolute amounts of isotope in the different positions of the glucose may be represented by the values for concentration, since atom per cent excess C^{13} × (mM of glucose)/100 = mM excess C^{13} for any given position in the glucose, and the factor (mM glucose)/100 is common to the calculation for all positions.

While the above figures may give an approximate indication of the relative amount of isotope that entered glycogen by different routes in the present studies, they need not indicate the relative amount of glycogen constituted from precursors coming over the different paths, since the degree of isotope dilution may not be the same for the different paths. Indeed, it seems quite reasonable to assume a lesser degree of dilution for isotope traversing the direct pathway of Reactions 2 and 1, from which it follows that the calculated ratios give a minimum value for the relative amount of glycogen formed from precursors that have been involved in the tricarboxylic acid cycle compared with those that have taken the direct path. In other words, a molecule of pyruvate introduced into the liver "pyruvate pool" should have, on the average, at least five chances to enter the reactions of the tricarboxylic acid cycle for every chance for entry into the glycolytic reactions (assuming that the reactions of glycolysis are essentially one way in the direction of glycogen deposition, under the circumstances obtaining). The recent paper of Topper and Hastings (15), in which the distribution of isotope in glycogen deposited in liver slices, with α-labeled pyruvate as substrate, is reported, is confirmatory, in general, of the corresponding experiments performed in the intact animal, as described in the present study and in a preliminary report (1). Topper and Hastings have calculated that involvement of pyruvate in CO_2 fixation and subsequent reactions occurs 4 times as rapidly as does entry into the reactions leading from pyruvate to glycogen. The presentation of the rôle of the tricarboxylic acid cycle in the distribution of isotope in glycogen is somewhat incomplete, since there is no discussion of the possibility that the cycle may act as a pathway, in addition to CO_2 fixation, for introducing isotope into positions 3 and 4 of the glucose. Also the possibility that there may be differences in the degree of isotope dilution due to side reactions occurring on the different metabolic pathways is not considered. It may be, of course, that this latter factor is of little importance in determining the results in experiments of this type. Its rôle, however, remains to be evaluated.

It should be pointed out that the foregoing discussion has assumed, implicitly, a homogeneous metabolic system in which mutual interaction between the different metabolic pathways could occur freely. This is obviously not the case in the intact rat. Nevertheless, it seems likely that, in the present experiments, the isotopic composition of liver glycogen reflects, in the main, metabolic events within the liver cell, where the assumption of homogeneity may more nearly apply. The isotopic composition of the respiratory CO_2, on the other hand, must result from the summed activity of all the tissues of the animal's body.

Finally, it should be pointed out that the general argument, presented in terms of current metabolic schemes, has been conducted in a rather dogmatic fashion in order to limit the discussion to a reasonable length. The general agreement between the results presented and these schemes enhances the likelihood of their reality in the intact animal, but cannot be considered as establishing them as the main or sole metabolic pathway involved in the present studies, as might be inferred if some sections of the discussion are construed too literally.

SUMMARY

$CH_3 \cdot C^{13}HOH \cdot COONa$, $C^{13}H_3 \cdot C^{13}HOH \cdot COONa$, and $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ have been fed to rats and the resulting liver glycogen isolated and degraded, and the distribution of isotope in the glucose chain determined. The results have been interpreted in terms of the reactions of glycolysis and the tricarboxylic acid cycle, with which schemes they appear to be in general agreement. The results with lactate labeled in the α- or β-carbon indicate that of the administered lactate carbon that was deposited as liver glycogen probably less than one-sixth entered the glycogen directly via the reactions of glycolysis, without prior passage through reactions involving randomization of isotope between the α- and β-carbons.

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CONVERSION OF PROPIONATE TO LIVER GLYCOGEN IN THE INTACT RAT, STUDIED WITH ISOTOPIC PROPIONATE*

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It has been found that when various fatty acids containing an odd number of carbon atoms in the chain (C_3, C_5, C_7, C_9 acids) are fed to fasted rats deposition of liver glycogen occurs, which is of approximately the same magnitude for each acid (4). The feeding of valeric, heptylic, pelargonic, and undecyclic acids to well fed rabbits leads to an increase in blood acetone bodies, while the administration of propionic acid does not (5). Similarly, incubation of the C_5, C_7, and C_9 acids with liver slices has been found to result in acetoacetate formation, which did not occur when propionate was used as the substrate (6). On the basis of these findings the odd carbon fatty acids have been considered as undergoing successive β oxidation, with the formation of propionate from the terminal 3-carbon residue. Recently Atchley (7) has demonstrated the formation of propionate in a kidney enzyme preparation, using valerate as the substrate. The fate of propionate itself in the animal body, however, is far from established. Propionate given to phlorhizinized dogs (8) and fasted rats (4) leads to the excretion of extra glucose and the deposition of liver glycogen, respectively. While these findings may be accepted as evidence for the conversion of propionate to carbohydrate, the chemical pathway for this transformation remains to be defined.

$$\underset{\text{Propionate}}{CH_3\cdot CH_2\cdot COO^-} \xrightarrow{-2H} \underset{\text{Acrylate}}{CH_2{:}CH\cdot COO^-} \xrightarrow{+HOH}$$

$$\underset{\text{Lactate}}{CH_3\cdot CHOH\cdot COO^-} \xrightarrow{-2H} \underset{\text{Pyruvate}}{CH_3\cdot CO\cdot COO^-}$$

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Hahn and Haarmann (9) have reported the accumulation of pyruvate in a muscle mince incubated with propionate and have suggested the accompanying series of reactions to account for their finding.

Bloch and Rittenberg (10), using deuterium as a labeling agent, have examined the ability of a variety of compounds to acetylate fed foreign amines. Propionate and alanine were studied, the latter being considered equivalent to pyruvate metabolically. On the basis of the deuterium content of the excreted acetyl groups, alanine was judged to be an efficient acetylating agent, while propionate was not. This was considered as evidence against the conversion of priopionate to pyruvate.

The present study, with use of carbon-labeled propionate, was undertaken in an effort to gain more information concerning the fate of propionate in the animal body. Variously labeled isotopic propionates, plus glucose, were fed to fasted rats, and, after a suitable period of time, the livers were removed, the glycogen isolated and hydrolyzed to glucose, and the distribution of isotope in the glucose chain determined. Although the observed isotope distribution patterns have been found to bear a basic resemblance to those obtained with similarly labeled lactate (11), a finding pointing to pyruvate as an intermediate in propionate metabolism, there are certain differences between the two sets of patterns which indicate that a direct conversion to pyruvate or lactate may not occur.

Methods

The following compounds were studied: $CH_3 \cdot CH_2 \cdot C^{13}OONa$, $C^{13}H_3 \cdot CH_2 \cdot COONa$, and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$. The carboxyl-labeled compound was synthesized by the Grignard reaction. The β-labeled propionate was prepared from $C^{13}H_3 \cdot COOH$ (3), and the C^{14},C^{13}-labeled compound from $C^{14}H_3 \cdot C^{13}OOH$ (11). The labeled acetates were reduced to ethanol by catalytic hydrogenation, then converted to ethyl iodide, and the corresponding propionate prepared via nitrile formation and hydrolysis (11). The partition coefficient (12) was determined for each of the three isotopic propionates and agreed with established values within experimental error. Conversion of the C^{14},C^{13}-propionate to lactate, and stepwise degradation of the lactate by well established procedures, showed the isotope to be distributed as anticipated from the synthetic methods employed (11).

The general plan of the experiments and the methods employed have been fully described in prior communications (13, 14, 11). In the experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, the glucose from the liver glycogen was degraded by the two types of procedure previously described (13).

RESULTS AND DISCUSSION

The general experimental data are summarized in Table I. In Table II are presented the results of the glycogen degradations. The data from the experiments with $C^{13}H_3 \cdot CH_2 \cdot COONa$ and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, when consideration is given to the known errors in the degradation methods (14), show that isotope originating in the α- and β-carbon of propionate is completely randomized between the 1,6 and 2,5 positions of glucose. The errors in the present degradation methods, as pointed out previously (14), are that fraction 1,6 is usually subject to contamination with a small amount of non-isotopic carbon, and that the fraction for carbon 3 is not entirely free of carbon from other positions. Carbon 6, from the methyl glucoside degradation, is obtained by a series of fairly specific reactions (13), and appears to be free from such contamination. On the assumption that the hexose chain is formed from 2 triose molecules of similar isotopic composition, carbon 6 may be taken as a measure of the isotope content of fraction 1,6. It will be noted that, in Experiments 7 to 10 and 11 to 13, both the C^{13} and C^{14} values for carbon 6 are in excellent agreement with the values obtained for carbons 2,5. Likewise there is good agreement between the experimentally determined isotope concentrations in the carbon 1, 2, 4, and 5 fraction of the methyl glucoside degradation and the values calculated for this fraction (*cf.* Table II). The over-all results are therefore taken to indicate that the isotope concentrations in the 1, 2, 5, and 6 positions are equal.

TABLE I

General Experimental Data from Rats

Experiment No.	Body weight after fast	Sex	Type of propionate administered	Amount of propionate administered per 100 gm. body weight	Amount of glucose administered per 100 gm. body weight	Liver weight	Liver glycogen (as glucose)
	gm.			*mM*	*mM*	*gm.*	*mM*
1	240	♂	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	2.60	2.32	7.2	0.19
2	210	♂	"	3.00	2.65	6.3	0.27
3	196	♀	$C^{13}H_3 \cdot CH_2 \cdot COONa$	2.12	3.53	4.6	0.56
4	182	♀	"	2.10	3.50	5.7	
5	188	♀	"	2.10	3.50	6.0	0.52
6	197	♀	"	2.11	3.51	6.7	0.23
7	210	♂	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	2.51	3.32	6.6	5.19
8	230	♂	"	2.54	3.37	7.0	
9	210	♂	"	2.54	3.37	7.3	
10	240	♂	"	2.66	3.52	8.4	
11	190	♂	"	2.70	3.54	6.6	3.98
12	220	♂	"	2.66	3.52	7.0	
13	230	♂	"	1.92	2.55	7.2	

Calculations of the per cent of administered isotope recovered in the

1, 2, 5, and 6 carbons of the glucose, presented in parentheses in the last column of Table II, yield values for C^{13} and C^{14} which are identical within experimental error, as might be expected for a mechanism of conversion of propionate to glucose which involved complete randomization of the α- and β-carbons of the propionate between the 1,6 and 2,5 carbons of the glucose.

TABLE II

Distribution of Isotope in Liver Glycogen after Administration of Labeled Propionate to Rats*

Experiment No.	Type of propionate administered	Type of isotope	Isotope concentration in labeled position of administered propionate	Isotope concentration in degradation fractions of glucose			Isotope concentration in degradation fractions of methyl glucoside			Per cent of administered isotope recovered in liver glycogen
				Carbon atoms of glucose			Carbon atoms of glucose			
				3,4	2,5	1,6	3	1,2,4,5	6	
1	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	C^{13}	4.62	0.15	−0.01					0.2
2	"	"	4.62	0.12	0.01					0.2
3- 6	$C^{13}H_3 \cdot CH_2 \cdot COONa$	"	2.28	0.04	0.17	0.16				2.64
7-10	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	C^{13}	6.50	0.09	0.38	0.35	0.09	0.32 (0.31)†	0.39	5.93 (5.31)‡
		C^{14}	11,600	183	683	645	198	548 (558)†	691	6.07 (5.35)‡
11–13	"	C^{13}	6.50	0.08	0.41	0.37	0.09	0.32 (0.33)†	0.41	7.17 (6.54)‡
		C^{14}	11,600	183	748	642	223	607 (607)†	746	7.50 (6.68)‡

* The C^{13} values are given in atom per cent excess, and the C^{14} values in counts per minute per mg. of carbon.

† The figures in parentheses are calculated values, in which the isotope concentration for carbon 4 is taken as equal to fraction 3,4 and that in carbons 1, 2, and 5 is taken as equal to fraction 2,5.

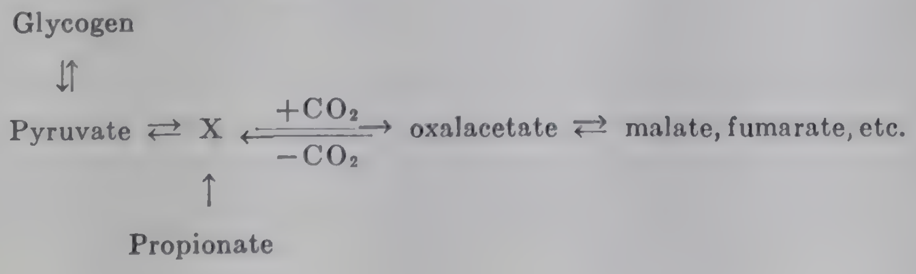
‡ The values in parentheses represent the per cent of administered isotope recovered in carbons 1, 2, 5, and 6, the isotope values for the 2,5 fraction being taken as representative of all 4 carbon atoms.

As previously reported (11), when $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ is fed to a fasted rat, the α- and β-carbons of the lactate are largely, though not completely, randomized between the 1,6 and 2,5 carbons of the glucose from the resulting glycogen. The distribution is, in general, the same as that noted in the present experiments with $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$, except that in the case of $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ C^{13} is present in somewhat higher concentration in carbons 2,5 than in carbons 1,6, while C^{14} is more abundant in the latter fraction than in the former. The incomplete randomization, in the case of lactate, has been explained in terms of the reactions of glycolysis and the tricarboxylic acid cycle. The fact that complete equalization does not occur has been ascribed to conversion to pyruvate and direct incorporation of some of the pyruvate into glycogen, prior to randomization of the isotope via the tricarboxylic acid cycle (11).

If one assumes that pyruvate is an obligatory precursor of glucose and glycogen, the isotope distribution patterns observed in the glycogen in the present studies indicate that propionate labeled in the α- or β-carbon gives rise to pyruvate in which both α- and β-carbons contain equal labeling.

In view of the results with labeled lactate (11), this may be taken to mean that propionate cannot be converted more or less directly to pyruvate (as, for example, via acrylate and lactate (9)), but that a more complex mechanism involving complete randomization of the α- and β-carbons intervenes. What this mechanism might be is a matter of conjecture. One possibility may be indicated in simplified form. Carboxylation on the methyl group of propionate would lead to symmetrical succinate. From such succinate (via fumarate, malate, and oxalacetate), pyruvate would be formed containing isotope from the α or β position of propionate equally distributed between both the α- and β-carbons. However, the authors are aware of no evidence for such a reaction in mammalian tissues. The reverse reaction, the formation of CO_2 and propionate by the decarboxylation of succinate, has been described in certain microorganisms (15–17). It is not meant to suggest that the statement, succinate → propionate + CO_2, describes anything but the net result of what may be a series of reactions. Similarly, it is intended only to point out that an over-all conversion of propionate to succinate by a $C_1 + C_3$ addition would account for the isotopic data (as well as for the glycogenic properties of propionate).

On the other hand, a more direct path from propionate to pyruvate may exist, but, because of differences in reaction rates, the major part of the propionate carbon traverses an indirect pathway to pyruvate, involving randomization of isotope. A hypothetical example is presented in the accompanying diagram.



Propionate is depicted as being converted without randomization to an intermediate, X, lying between pyruvate and the reactions of the tricarboxylic acid cycle. If the reaction rate from X toward the randomizing reactions of the tricarboxylic cycle is sufficiently greater than that from X toward pyruvate, isotope from the α- or β-carbons of propionate could be fairly completely randomized between the α and β positions of the pyruvate formed from X. The only available evidence bearing on this suggestion is the finding by Utter and Chenoweth (18) that pyruvate may not be the immediate reactant involved in the CO_2 fixation reaction by which oxalacetate is formed.

TABLE III

Data on Respiratory CO_2 of Rats

Experiment No.	Type of propionate administered	CO_2 output in mM[*]			Type of isotope	Isotope concentration[†] in respiratory CO_2			Per cent of administered isotope recovered in respiratory CO_2
		1st period	2nd period	3rd period		1st period	2nd period	3rd period	
1	$CH_3 \cdot CH_2 \cdot C^{13}OONa$	8.7	8.5	7.9	C^{13}	0.12	0.21	0.23	16.6
2	"	7.2	6.2	6.3	"	0.13	0.25	0.27	13.1
3–4	$C^{13}H_3 \cdot CH_2 \cdot COONa$	12.4	12.2	14.8	"	0.08	0.08	0.10	19.3
5–6	"	13.7	14.2	13.9	"	0.06	0.09	0.08	17.4
7	$C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$	8.9	8.5	8.6	"	0.12	0.22	0.26	15.2
					C^{14}	232	473	518	17.3
8	"	7.5	8.5	8.6	C^{13}	0.13	0.17	0.07	7.9
					C^{14}	218	369	188	9.4

[*] The CO_2 collection periods were 50 minutes, except for Experiments 7 and 8, in which they were 1 hour.

[†] See foot-note to Table II.

It is interesting to note that the degree of isotope dilution undergone by the $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$ in its conversion to liver glycogen is of the same order as that found for $C^{14}H_3 \cdot C^{13}HOH \cdot COONa$ (11). This together with the observed isotope distribution patterns indicates that propionate carbon gives rise to pyruvate (assuming pyruvate to be an obligatory intermediate in glucose and glycogen formation), and at about the same rate as lactate. That the findings of Bloch and Rittenberg using deuterium-labeled propionate in isotope dilution experiments (10), as pointed out earlier, are not in agreement with the foregoing conclusion may have been due to the loss of deuterium from the propionate in the same reactions which bring about the complete randomization of the α- and β-carbons of propionate in the 1, 2, 5, and 6 positions of the glucose in the present experiments.

Some of the other experimental results obtained in the present study are worthy of note. The data for respiratory CO_2 in Experiments 7 and 8 (Table III) indicate that the α- and β-carbons of propionate are converted to CO_2 at approximately equal rates. Although the differences between the rate of recovery of C^{13} and C^{14} in the respiratory CO_2 appear fairly large, they are not significant because of the relatively larger error of the isotopic measurements on the respiratory CO_2 samples, owing particularly to the low C^{13} levels in the respiratory CO_2. It may be noted, incidentally, that the glycogen degradation data in the experiments with $CH_3 \cdot CH_2 \cdot C^{13}OONa$ and $C^{13}H_3 \cdot CH_2 \cdot COONa$ rule out β oxidation to malonate with the formation of acetate (or an equivalent 2-carbon compound) from the α- and carboxyl carbons of malonate as an important pathway in propionate metabolism. Since both types of propionate would yield carboxyl-labeled malonate, and consequently a carboxyl-labeled 2-carbon compound, this mechanism should deposit isotope exclusively in positions 3 and 4 of the glucose from liver glycogen following feeding of both types of propionate (14). The finding of label predominantly in the 1,6 and 2,5 carbons in the experiments with the β-labeled propionate cannot be reconciled with this pathway.

It should be clear that the foregoing discussion cannot be generalized to extrahepatic tissues, in which reactions may occur that will not affect the isotope distribution in liver glycogen. In this connection it should be pointed out that in many instances in the present and in former experiments with other labeled compounds glycogen of cardiac and skeletal muscles has been isolated and degraded. Isotope concentrations have always been much lower than those found in liver glycogen, but the distribution patterns have always been the same. While this may indicate basically similar metabolic pathways in liver and muscle for the compounds concerned, it may also be nothing more than a reflection of hepatic metabolism via the blood sugar.

SUMMARY

Isotopic propionate ($CH_3 \cdot CH_2 \cdot C^{13}OONa$, $C^{13}H_3 \cdot CH_2 \cdot COONa$, and $C^{14}H_3 \cdot C^{13}H_2 \cdot COONa$) has been fed to fasted rats and the resulting liver glycogen isolated, hydrolyzed to glucose, and degraded. Isotope from the carboxyl group of propionate has been found to give detectable labeling only in carbon atoms 3 and 4 of the glucose, while propionate containing isotope in the α or β position gives rise to glucose labeled predominantly and equally in carbons 1, 2, 5, and 6.

If it is assumed that pyruvate is an obligatory intermediate in glucose and glycogen formation, the results indicate that propionate gives rise to pyruvate, but that in the process the α- and β-carbons of the propionate

lose their orientation and become equally distributed between the corresponding groups of pyruvate. Some possible explanations for this finding are advanced.

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THE EFFECTS OF TRYPTOPHAN DEFICIENCY UPON ENZYME ACTIVITY IN THE RAT*

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Recently it has been observed that liver enzyme activity may be markedly affected by dietary conditions. Seifter *et al.* (1) have demonstrated that during complete dietary protein deprivation loss of arginase and D-amino acid oxidase from rat liver occurs more rapidly than loss of general liver protein. We (2) have observed that the level of xanthine oxidase activity in rat liver may be altered by subtle protein changes in the ration. Adult rats fed a diet completely adequate for maintenance showed a decreased liver xanthine oxidase content which was returned to normal by addition of methionine to the ration. It appeared that liver xanthine oxidase activity was a fairly sensitive index of dietary amino acid availability.

In a previous study of the effects of methionine deficiency upon liver enzyme activity, it was noted[1] that complete deprivation of dietary methionine reduced liver xanthine oxidase activity almost completely, decreased succinic oxidase activity somewhat, and had no demonstrable effect upon liver endogenous respiration.

In the present paper the study of the effects of amino acid deficiencies upon liver enzyme activity has been extended to tryptophan. By studying the relation of individual amino acid deficiencies to liver enzyme activity, it is hoped that some light will be thrown upon amino acid, enzyme, and animal protein metabolism in general.

The enzyme systems we have chosen for study in this work are liver xanthine oxidase, succinic oxidase, cathepsin, endogenous respiration, and fatty acid oxidase. Since xanthine oxidase activity has been observed to be very sensitive to dietary protein changes, this enzyme was one of those chosen for the present study. The possibility that increased breakdown of body proteins during a tryptophan deficiency might involve a greater need for liver protease activity led us to study liver catheptic activity as a

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[1] Williams, J. N., Jr., Denton, A. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **72**, 386 (1949).

function of tryptophan deficiency. Since endogenous respiration of the liver may be taken as a measure of general liver metabolic rate, the effects of lack of dietary tryptophan upon this factor were studied. Both succinic oxidase and fatty acid oxidase have been shown by Schneider (3) and Kennedy and Lehninger (4), respectively, to be centered in the cell mitochondria. Therefore, deficiency studies of these enzymes might give some evidence concerning metabolism of the particulate cell matter.

EXPERIMENTAL

Adult male rats of the Holtzman strain weighing from 290 to 310 gm. were divided at random into two groups and placed in individual cages. One group was given a complete ration and the other a tryptophan-free ration for at least 2 weeks before being used in the enzyme studies. Both groups were fed and watered *ad libitum* throughout the feeding period. The complete ration has been described in a previous communication (2). Briefly it consisted of acid-hydrolyzed casein, plus a mixture of purified amino acids supplementing those destroyed during acid hydrolysis, 18 per cent; Salts IV (5) 4 per cent; a mixture of the known vitamins in sucrose base 2 per cent; corn oil 5 per cent; and sucrose to make 100 per cent. The tryptophan-free ration was the same as that described, except that DL-tryptophan was omitted from the mixture of amino acids. The acid-hydrolyzed casein was shown by microbiological assay to be devoid of tryptophan. During the feeding period records were kept of amounts of food consumed by each animal. After the tryptophan-deficient animals had lost approximately 25 per cent of their body weight, both groups of animals were sacrificed for the enzyme determinations. At the time of sacrifice the control animals weighed 330 ± 10 gm.,[2] while the tryptophan-deficient rats weighed 230 ± 5 gm.[2]

The animals were stunned by a blow on the head and decapitated. The livers were removed and chilled in cracked ice. Aliquots of the chilled livers were weighed, mixed with known volumes of cold buffer or water (depending upon the enzyme system to be studied), and homogenized. Aliquots of the homogenates were removed for total nitrogen, dry weight, and non-protein nitrogen determinations. Non-protein nitrogen was measured by precipitating the liver proteins in 10 per cent trichloroacetic acid, filtering, and determining nitrogen in aliquots of the filtrate. Difference in total nitrogen and non-protein nitrogen gave homogenate protein nitrogen, which, when multiplied by 6.25, gave liver protein per ml. of homogenate. Approximately 0.1 gm. portions of the unhomogenized livers were analyzed for total nitrogen both to serve as a check on the accuracy of preparing the homogenates and to give a better comparison of total liver nitrogen between animals. All nitrogen determinations were carried out in duplicate by a semimicro-Kjeldahl procedure.

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[2] Standard error of the mean.

Xanthine oxidase activity was determined according to the method of Axelrod and Elvehjem (6). Succinic oxidase activity was measured by the manometric method of Schneider and Potter (7). Catheptic activity was determined according to a modification of Anson's method, as outlined by Miller (8). Oxygen uptake during the first 10 minutes before the xanthine substrate was added in the xanthine oxidase determinations was taken as a measure of endogenous respiration of the livers. Fatty acid oxidase activity was assayed by a slight modification of Lehninger's method (9). All enzyme activities were determined at 30°.

TABLE I

Effects of Tryptophan Deficiency on Enzyme Activity in Rat Liver

Enzyme determined	Ration	No. of animals	Enzyme activity*		
			Based on liver protein	Based on liver dry weight	Based on liver wet weight
Xanthine oxidase	Complete	7	1.1 ± 0.2†	0.50	0.141
	Tryptophan-free	6	0.49 ± 0.04	0.27	0.074
Succinic oxidase	Complete	10	109 ± 5	51	2.50
	Tryptophan-free	7	87 ± 3	47	2.19
Cathepsin	Complete	8	98 ± 4	53	15
	Tryptophan-free	8	99 ± 5	52	15
Endogenous respiration	Complete	7	8.8 ± 1.0	5.6	1.37
	Tryptophan-free	6	3.5 ± 0.4	1.9	0.52
Fatty acid oxidase	Complete	4	16.4 ± 0.9	11.2	
	Tryptophan-free	4	16.0 ± 1.0	11.2	

* For individual enzyme units see the text.

† Standard error of the mean.

RESULTS AND DISCUSSION

It was observed during the feeding period that both groups of animals consumed approximately the same amount of ration. Therefore, any effects of protein starvation due to decreased food intake after the onset of the amino acid deficiency were negligible. A true tryptophan deficiency was present in the animals of that group, as evidenced by rapid loss of weight and extreme nervousness of the animals.

Results of the enzyme determinations are presented in Table I. Standard errors of the means are reported only for enzyme activity based on liver protein, although approximately the same relative deviations hold for the other results. The enzyme activities are reported in terms of liver protein, liver dry weight, and liver wet weight, since dilution of the liver by lipide or glycogen might cause erroneous conclusions to be drawn from the enzyme results. Enzyme activity in terms of liver protein negates those effects.

Xanthine oxidase activity is expressed in microliters of oxygen uptake per hour per gm. of liver protein, dry liver, and fresh liver. Succinic oxidase activity is reported as microliters of oxygen uptake per hour per mg. of liver protein, dry liver, and fresh liver. Units of cathepsin activity are defined as gm. of hemoglobin nitrogen made soluble per 3 hours per gm. of liver protein, dry liver, and fresh liver. Endogenous respiration is expressed as microliters of oxygen uptake per hour per mg. of liver protein, dry liver, and fresh liver. Fatty acid oxidase is measured with octanoate as substrate in terms of microliters of oxygen uptake per hour per mg. of liver protein and dry liver. Calculation on the basis of fresh weight of liver could not be made in this case, since only the centrifugable portion of the homogenates was employed.

From Table I it can be seen that liver xanthine oxidase activity again appeared to be highly labile under adverse dietary protein conditions. A tryptophan deficiency, however, did not reduce activity of this enzyme to zero as in a methionine deficiency.[1] Succinic oxidase activity was also decreased faster than other liver protein in a tryptophan deficiency, although disappearance of activity of this enzyme was not nearly as fast as that of xanthine oxidase. It is perhaps significant that the ratio of enzyme activities of the two groups of animals when based on liver protein is different from the ratio when the activities are based upon liver, dry weight or fresh weight. The livers of the tryptophan-deficient animals were not noticeably infiltrated with fat as in a methionine deficiency. From Table II it can also be seen that in the tryptophan deficiency no detectable decrease in liver protein per gm. of liver occurred.

Catheptic activity of livers of the tryptophan-deficient animals appeared to be conserved during the deficiency period. Miller (8) observed that during acute inanition in the rat activity of this enzyme is retained more tenaciously than other liver protein. The reasons for conservation of this enzyme are not clear. However, the fact that in our results catheptic activity is lost at the same rate as general liver protein is significant.

It has frequently been reported from this laboratory that dietary conditions appear to have little effect upon liver endogenous respiration. In the present results, however, there was a sharp decrease in endogenous respiration in a tryptophan deficiency. This may be due to the possible conversion of the tryptophan to coenzymes I and II via nicotinic acid. This appears doubtful, however, since both groups of animals received the same adequate level of nicotinic acid in the diet. It is possible that activities of enzymes involved in endogenous respiration are decreased in a tryptophan deficiency in a similar fashion to xanthine oxidase activity. If this is so, tryptophan must be very specific for that action, since a methionine deficiency[1] and various vitamin deficiencies (10, 11) have little effect upon liver endogenous respiration.

The complex fatty acid oxidase system appeared to be essentially unaffected by a tryptophan deficiency. Although both fatty acid oxidase and succinic oxidase are centered in the cell mitochondria, activity of one may be retained by the liver and activity of the other lost.

In Table II are reported general characteristics of livers of animals of the two groups. Although the tryptophan-deficient animals lost weight rapidly, the protein proportion of the liver was not significantly altered. It has been reported by Seifter *et al.* (1) and others that during total protein deprivation nitrogen per gm. of liver is measurably decreased. It is probable that during a tryptophan deficiency the conditions of negative nitrogen balance are less drastic than complete protein starvation and the ratio of liver protein to liver water, glycogen, and lipide was not noticeably changed. This is upheld by the fact that the per cent of liver dry weight was unchanged by the tryptophan deficiency. It might be expected that non-protein nitrogen of the liver would be increased in the deficient animals. As the results indicate, however, this liver factor remained constant in the tryptophan deficiency.

TABLE II

Effects of Tryptophan Deficiency on General Liver Characteristics

Liver component measured	Ration	No. of animals	Component per 100 gm. liver
			per cent
Protein	Complete	18	16.2 ± 0.5*
	Tryptophan-free	18	16.2 ± 0.4
Non-protein nitrogen	Complete	18	0.29 ± 0.003
	Tryptophan-free	18	0.28 ± 0.01
Dry weight	Complete	23	28.2 ± 0.4
	Tryptophan-free	20	28.1 ± 0.2

* Standard error of the mean.

From the results of these experiments it appears that a tryptophan deficiency in the rat is sufficiently drastic to cause a marked loss in activities of certain enzyme systems. The effects of the deficiency upon enzyme and general liver characteristics, however, appear to be different both qualitatively and quantitatively from the effects of inanition, protein deprivation, and methionine deficiency. The effects of other amino acid deficiencies upon enzyme systems are being studied by the authors at present. It is hoped that any differences in metabolic effects of the individual amino acid deficiencies will be brought out by these studies.

SUMMARY

1. A tryptophan deficiency in the rat reduces markedly liver xanthine oxidase activity and endogenous respiration. Succinic oxidase is also noticeably decreased. Activities of cathepsin and fatty acid oxidase are not altered by this deficiency condition.

2. General liver characteristics such as liver protein, total solids, and non-protein nitrogen per gm. of liver remain unchanged in a tryptophan deficiency.

3. Qualitative and quantitative interrelationships of a tryptophan deficiency, complete inanition, protein deprivation, and methionine deficiency in the rat to liver enzyme activity and other liver factors have been discussed.

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FREE AMINOETHYLPHOSPHORIC ESTER IN RAT ORGANS AND HUMAN TUMORS*

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In the course of an investigation on the free amino acids of rat organs by means of paper chromatography, several compounds were detected which could not be identified with any of the known amino acids. One component was always found in relatively large amounts in the spleen and in several human tumors (1). The position of this unknown component corresponded closely with aminoethylphosphoric ester in the map of spots of Dent (2). A similar or perhaps identical substance was reported by Work (3) in hydrolysates of *Corynebacterium diphtheriae.* She attempted to identify it by means of alkaline phosphatase, but her results were negative. No details of her procedure were given.

The results presented in this paper show that the unknown substance found in spleen and tumors is in fact aminoethylphosphoric ester. The analyses were extended to twenty-three organs of the rat and five human tumors. In every case aminoethylphosphoric ester was found in variable concentrations.

Methods

The extracts of rat organs were made according to a procedure described by Awapara (4). Tumor specimens were obtained from surgery and processed immediately after removal to avoid any possible decomposition. Chromatographic analyses were carried out with phenol and 2,4-lutidine as solvents. Quantitative determination of the fractions was made by the method of Landua and Awapara (5).

The synthesis of aminoethylphosphoric ester was carried out according to the procedure given by Plimmer and Burch (6).

EXPERIMENTAL

The presence of the unknown compound in beef spleen was first established by means of paper chromatography. It was shown that the substance from beef spleen was identical with the substance present in rat spleen and in human tumors, in so far as chromatographic behavior is concerned.

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Partial Fractionation of Beef Spleen—About 1 kilo of fresh beef spleen was cut into small pieces, covered with water, acidified with acetic acid, and heated for a couple of hours. The broth was filtered through a large Büchner funnel. The residual pieces of tissue were homogenized in a Waring blendor with hot acidified water. The pulp was filtered and the resulting filtrate combined with the first one. The combined filtrates were evaporated on a water bath to a small volume. After cooling, the syrupy extract was treated with sufficient ethanol so that the final alcohol concentration would be 90 per cent. A heavy white yellowish precipitate was formed. The precipitate was separated by centrifugation, followed by one washing with 80 per cent ethanol. Samples of the precipitate and supernatant were examined by paper chromatography. The major portion of the unknown substance was found in the precipitate accompanied by other compounds which gave positive ninhydrin reaction.

Isolation of Unknown Fraction—Attempts were made to isolate the unknown fraction by means of specific adsorbents. Lloyd's reagent removed most of it from the solutions of the crude extract, but recoveries from the column were very low and heavily contaminated. Acid-treated alumina was also found effective in removing the unknown, but again the recoveries were low. The main contaminants in every case were aspartic and glutamic acids. Removal of the dicarboxylic acids by the Foreman method was tried and the unknown substance precipitated nearly quantitatively with the acid.

Paper chromatography itself was tried out as a method for isolating small amounts of the unknown substance. The capacity of filter paper is not very large, but sufficient material was isolated by using the following procedure: An aqueous solution of the alcoholic precipitate was made to contain 1 gm. in 10 ml. The clear solution was applied along a line on one edge of a large sheet of filter paper, Whatman No. 4, in such a way as to form a thin line. After drying, the application was repeated. In this manner, about 1 ml. of solution could be applied on one sheet of paper. Ten such papers were prepared and chromatography carried out with phenol for 30 hours. A 2 cm. strip was cut from the dried chromatogram and developed with ninhydrin. With the strip as a guide, the section corresponding to glutamic acid was cut and eluted with water. The unknown overlapped with glutamic acid in the phenol run so that a second resolution became necessary. The eluates from all ten chromatograms were pooled and evaporated to a small volume in the water bath. Chromatography of this solution was carried out with 2,4-lutidine in the same manner as described above. Glutamic acid and the unknown were completely resolved by this procedure. The unknown material was eluted out of the paper in fairly pure state. No other ninhydrin-reacting substance could be found on chromatography with several different solvents.

Identification of Unknown Fraction—Aliquots of the paper eluate were hydrolyzed with 6 N hydrochloric acid and 20 per cent sodium hydroxide for 24 hours in sealed tubes. Acid hydrolysis caused no change in the chromatographic behavior of the unknown. Alkaline hydrolysis, however, brought about some changes, but no new spots were detected. Tests for phosphoric acid were carried out by the Fiske and Subbarow procedure. The alkaline hydrolysates gave a strong positive test, whereas the unhydrolyzed sample was negative. The chromatographic behavior of the unknown was not changed by oxidation with 30 per cent hydrogen peroxide. With this information on hand, it seemed highly probable that the unknown fraction was indeed aminoethylphosphoric ester.

TABLE I

Approximate Concentration of Aminoethylphosphoric Ester in Rat Organs and Human Tumors

The results are given in mg. per 100 gm. of fresh tissue.

Rat organ		Rat organ	
Liver (5)	17	Salivary gland (2)*	19
Kidney (5)	Trace	Thymus (adult) (6)*	90
Muscle (5)	20	" (young) (4)*	88
Heart (5)	20	Pituitary (35)*	45
Spleen (5)	116	Thyroid (37)*	7
Brain (5)	12	Adrenals (25)*	40
Ileum (5)	6	Pineal body (87)*	Present
Peyer's patches (5)	27		
Colon (5)	28	Human tumors	
Pancreas (5)	100		
Lymph nodes (5)*	105	Skin carcinoma	29
Ovary (3)	60	Breast carcinoma	58
Uterus (4)	15	Hepatoma	24
Testis (5)	29	Ovary carcinoma	37
Seminal vesicle (5)*	9	Spleen, leucemia	55
Coagulating gland (7)*	6		

The figures in parentheses represent the number of animals.

* The samples were pooled.

The final proof was furnished by the identification of ethanolamine in the hydrolysate. Because of the resistance of ethanolamine phosphoric ester to acid hydrolysis, it was necessary to carry out the reaction with 6 N hydrochloric acid in a sealed tube for 45 hours. At the end of this period, sufficient ethanolamine was released for identification by means of paper chromatography. Known solutions of ethanolamine were treated with hydrochloric acid in the same manner as the unknown. Chromatography of both solutions gave identical spots.

Further proof was obtained by chromatography of a mixture of the unknown substance with synthetic aminoethylphosphoric ester. In all solvents tried, a single homogeneous spot was obtained.

Incubation of the eluate with rat kidney extracts brought about complete disappearance of the aminoethylphosphoric ester.

RESULTS AND DISCUSSION

The fraction identified as aminoethylphosphoric ester in beef spleen was found to occur in nearly every organ of the rat and in several human tumors. The relative concentration in each organ is given in Table I. The values reported probably do not represent the absolute values because of the insolubility of aminoethylphosphoric ester in alcohol. However, all values are equally obtained and give an index of the relative concentration in each organ.

The results of this investigation indicate that aminoethylphosphoric ester is a widely distributed metabolite. Outhousen (7, 8) isolated the ester from several tumors but failed to detect it in various normal organs examined. Using improved methods of isolation, Colowick and Cori (9) presented evidence of the existence of aminoethylphosphoric ester in pig and rabbit intestine. Chargaff and Keston (10) determined the rate of phosphatide formation *in vivo* from aminoethylphosphoric ester containing the radioactive isotope of phosphorus. Their results suggested that tumors belong to the most active tissues with regard to the formation of phosphatides from aminoethylphosphoric ester.

SUMMARY

1. Aminoethylphosphoric ester was identified in twenty-three organs of the rat and several human tumors. This substance was isolated from beef spleen by means of paper chromatography and identified by the same means.

2. Approximate values for the concentration of aminoethylphosphoric ester in organs of the rat and in tumors are given.

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METABOLIC STUDIES OF AZOTOBACTER AGILIS BY THE USE OF A MUTANT DEFICIENT IN PYRUVIC OXIDASE*

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An account has been given elsewhere of the successful isolation of physiological x-ray mutants of *Azotobacter agilis* with genetic blocks in the substrate oxidation mechanisms (1). The present paper deals in greater detail with a mutant strain, designated A13, that was isolated as a variant unable to grow on glucose, and gives the methods used to characterize the particular deficiency responsible for this alteration.

Materials and Methods

The source of the organism, as well as the methods of culture and the media, has been described in previous publications (1, 2). Strain A4.4 is the original organism and strain A13 is a mutant isolated from an x-ray-treated culture.

The methods for the respiration experiments with cell suspensions have also been published (2), except for the slight modification for carbon dioxide determination. This was accomplished by setting up a series of identical Warburg cups without alkali in the center well and releasing the carbon dioxide from the neutral medium with acid at different stages. The oxygen consumption was determined at the same time in a cup with KOH and the amount of carbon dioxide computed from the difference. In the particular case in question the rate of oxygen uptake was shown to be independent of the presence of CO_2.

Pyruvic acid was estimated by Lu's colorimetric method as modified by Friedemann and Haugen, with toluene as the extraction solvent (3). The same method, with ethyl acetate instead of toluene, was employed for total keto acid determinations. Since the light absorption curves for the 2,4-dinitrophenylhydrazones of pyruvate and oxalacetate are identical (3) and these two acids were the only keto acids present, this estimation coupled with the pyruvate determination could be used to determine oxalacetate.

cis-Aconitic acid was synthesized from *trans*-aconitic acid by the method of Malachowski and Maslowski (4).

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* The material presented in this paper is taken from a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of California in June, 1947.

Results

It has already been reported (1) that strain A13 is completely unable to develop on the following compounds which are utilized for growth by the parent strain: pyruvate, lactate, malate, fumarate, succinate, α-ketoglutarate, *cis*- and *trans*-aconitate, tartrate, glucose, fructose, and gluconate. Growth of the mutant is supported only by ethyl alcohol, acetic acid, and malonic acid, and on these three substrates, it is indistinguishable from that of the original strain. Neither organism will grow on mannitol, galactose, xylose, D-arabinose, 2-ketogluconate, glycerol, citrate, diacetyl, propionate, oxalate, formate, glutamate, aspartate, alanine, or glycine. Both are able to adapt themselves to butyrate, apparently by a mutation mechanism, since only a few colonies develop. This change does not affect utilization of other substrates.

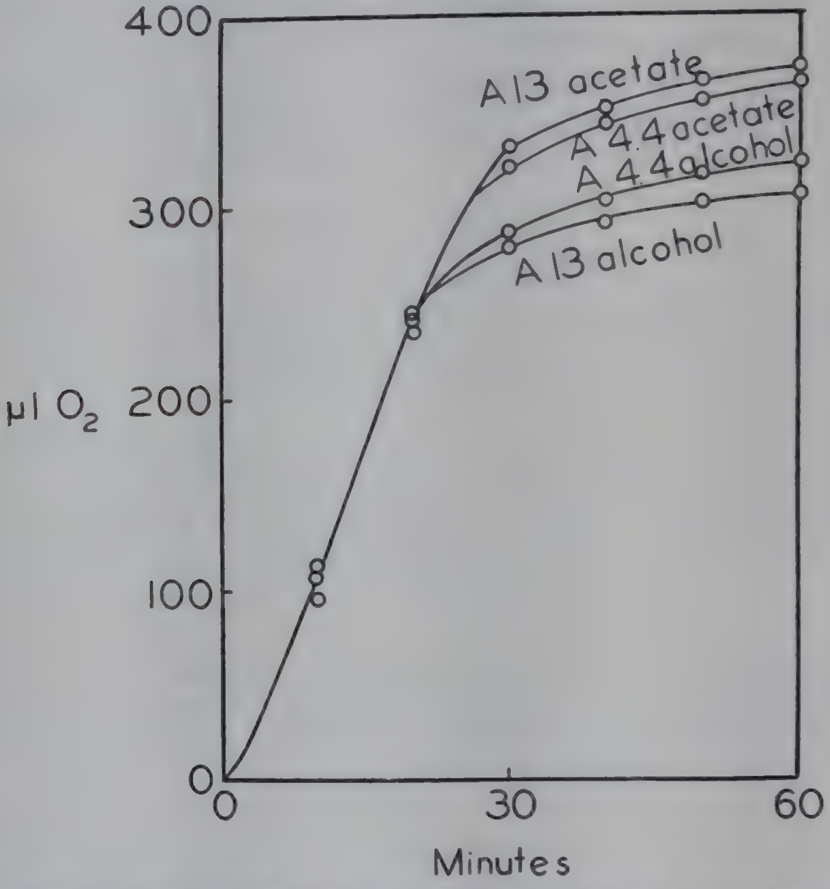


FIG. 1. Respiration curves for strains A4.4 and A13 on alcohol and acetate. Theoretical oxygen uptake for complete oxidation, 448 μl. of O_2.

The two strains exhibit identical behavior, not only in regard to growth but also as concerns respiratory rates, when tested on ethyl alcohol or acetate. This is illustrated in Fig. 1. Certain compounds which support growth of strain A4.4, but not of strain A13, are still oxidized by the latter, as shown in Fig. 3. For comparison the respiratory curves for the parent strain on the same compounds are given in Fig. 2. The same quantity of cells was used in the experiments shown in Figs. 2 and 3.

It will be noted on the respiration curves for strain A13 metabolizing the 4-carbon dicarboxylic acids that the rate is always slow and that a distinct break occurs when less than the theoretical amount of oxygen for complete oxidation has been consumed (Fig. 3). The same is true for α-ketoglutarate, which has a much longer adaptation period. Pyruvate is

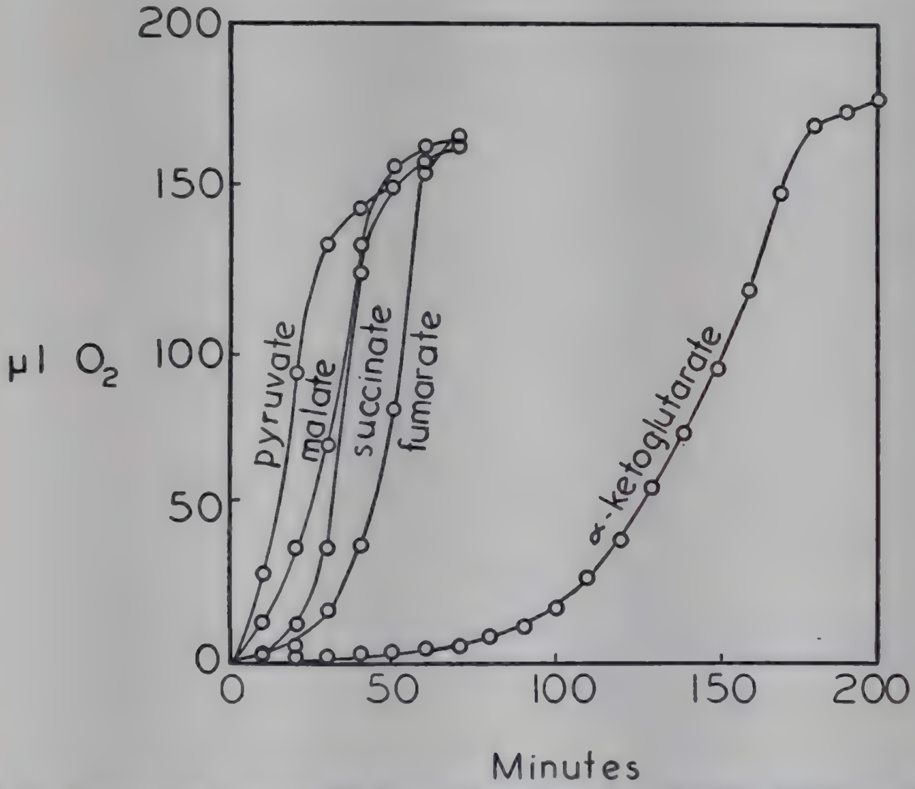


Fig. 2. Respiration curves for strain A4.4, grown on alcohol. Theoretical oxygen uptake for all substrates, 224 μl. of O_2.

the only slowly oxidized compound which is used at a constant rate, and the oxygen consumption for the other substances, after the break, levels off to that same rate. The oxygen taken up before the inflection corresponds to the amount required for oxidation to the pyruvate stage. The breaks occur at different times, and the total amount of oxygen consumed at these points is the sum of that used to convert the substrate to pyruvate and that subsequently used up in its oxidation. It should require 5 μM or 112 μl. of oxygen to convert 5 μM of succinate to the oxidation state of pyruvate, while fumarate and malate require only half that amount. If we allow for experimental error, it is evident that the distances from the pyruvate curve to the other curves on Fig. 3, after the breaks have occurred, are in this order of magnitude. A comparison of the CO_2 curve with the oxygen curve of malate (Fig. 4) shows that very little carbon has been split off before the break, suggesting that oxalacetate rather than pyruvate accumulates as an intermediate product during the oxidation. This is confirmed by the data in Table I which show the formation of keto acids during the oxidation of malate. The difference between the total keto acids and pyruvate is taken as oxalacetate. Isolation of the keto acid by the method of Clift and Cook (5) was unsuccessful, a syrup being obtained instead of crystals. But the light absorption curve for this derivative was identical with that of oxalacetate. Furthermore, the compound was decomposed to form the theoretical amount of carbon dioxide by the aniline citrate method. This leaves little doubt that it is actually

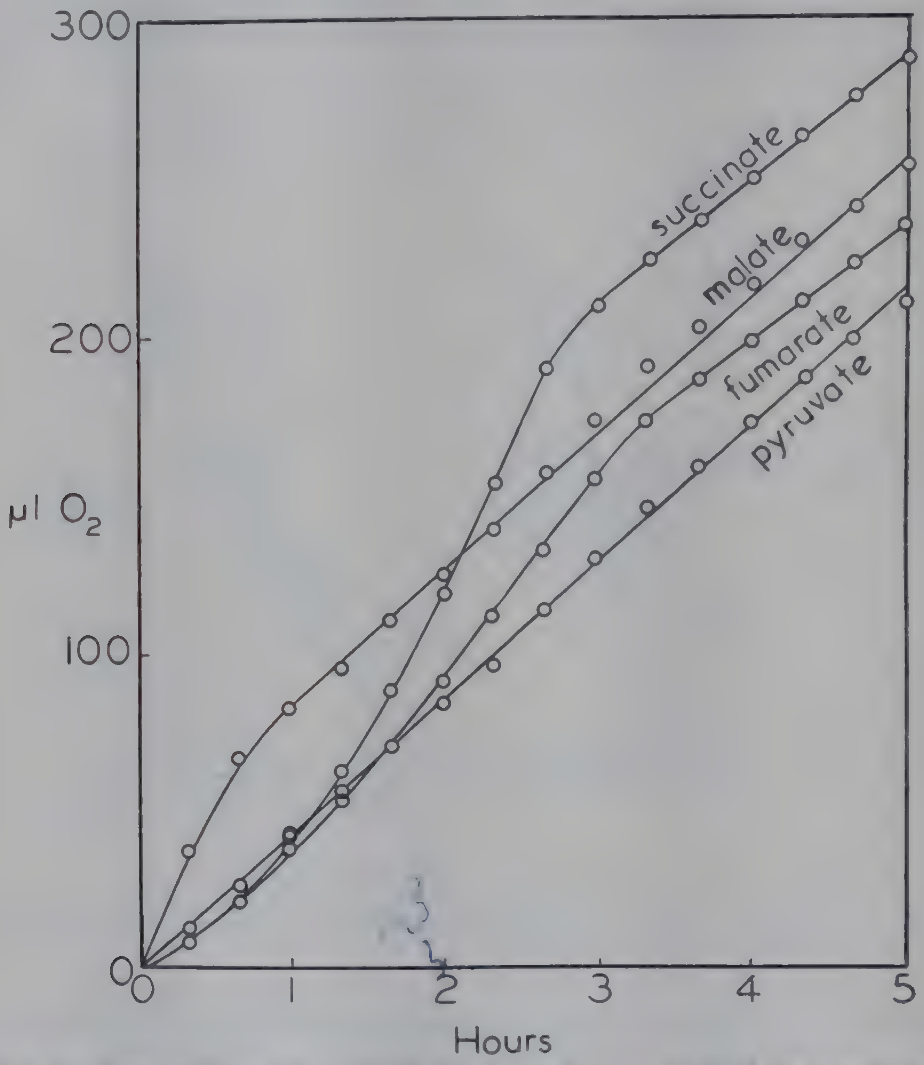


FIG. 3. Respiration of strain A13, grown on alcohol. 5 μM of each substrate

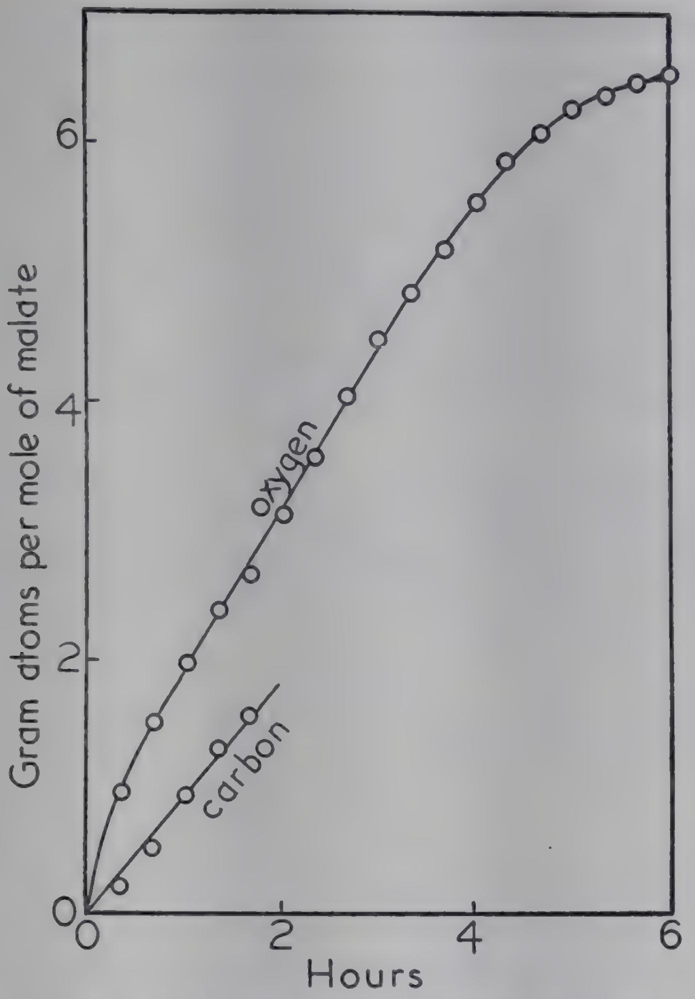


FIG. 4. Oxygen uptake and carbon dioxide production by strain A13 on malate. Theory for complete oxidation, 6 gm. atoms of oxygen and 4 gm. atoms of carbon per mole of malate.

TABLE I

Keto Acids Formation during Malate Oxidation by Strain A13

Initial malate, 5.00 μM. The break on the O_2 uptake curve occurred at 20 minutes (see Fig. 4).

Time	Oxalacetate	Pyruvate	Total
min.	*μM*	*μM*	*μM*
0			0.04
20	3.89	0.75	4.64
60	3.04	1.96	5.00

oxalacetate. In Table I the value for total keto acids is somewhat higher than that expected after 1 hour, probably owing to an experimental error. But in any event, the evidence is fairly conclusive that the mutant strain has a metabolic block in the oxidation of pyruvate and oxalacetate.

Further experiments were undertaken to find out whether this disturbance was the only metabolic effect of the mutation. Certain compounds such as glucose can be decomposed by the parent strain, but not by the mutant, within the time limit of these experiments. All these substrates have one thing in common: they always require a long adaptation period before they are attacked by alcohol-grown cells. Since the mutant shows in general a slower rate of adaptation than the normal strain when exposed to compounds above the block, it would not be expected to be able to adapt itself within the time limit, since it is not entirely stable and reverts to the parent type on exposure to the new substrate for a day or longer.

The rates of attack on compounds which could be conveniently studied were compared in the following manner. Strains A4.4 and A13 were grown in 0.2 per cent alcohol. After growth had ceased due to depletion of the alcohol, 1 per cent succinate was added, and incubation was continued on a shaker overnight. The cells, which should have become adapted to succinate and its breakdown products, were harvested and used for a Q_{O_2} (N) determination. The rate during the second 10 minute interval following substrate addition was used in the calculation.

TABLE II

Respiratory Rates for Succinate-Adapted Cells, Expressed As Q_{O_2} *(N)* $\times$ *10^{-3}*

	Observed rates		Computed initial rates	
	Strain A4.4	Strain A13	Strain A4.4	Strain A13
Succinate	10.7	2.0	1.5	0.9
Fumarate	7.6	0.9	1.3	0.7
Malate	11.6	10.8	1.9	10.6
Pyruvate	8.2	0.2	1.6	0.04
Acetate	15.8	16.3	4.0	4.1

The resulting respiratory rates are shown in the first two columns of Table II. Since these are over-all rates for reactions, some of which lead to accumulation of an intermediate, their significance is not immediately apparent. The rate of oxygen uptake with a given substrate by two organisms may differ greatly because of differences in the completeness of oxidation, even though the initial rate is the same. To overcome this difficulty, the rate at which the first step proceeds in the oxidation of each substrate was computed. Since there is no evidence of breaks on the oxygen curves for succinate, fumarate, and malate obtained with strain A4.4, the rate for the initial step should be the over-all rate divided by the number of oxygen atoms required for complete oxidation. The same is true for the oxidation of pyruvate and acetate by both strains. For strain A13, when a break occurs at the pyruvate stage, the initial rates for succinate, fumarate, and malate are the observed rates less the rate for pyruvate divided by the number of oxygen atoms required before the pyruvate stage. As an example, the observed rate for strain A13 on succinate is 2.0 ml. per hour per mg. of cell N. The rate for the oxidation to the pyruvate stage is this less the observed rate for pyruvate (0.2), or 1.8. 2 oxygen atoms are required to convert succinate to pyruvate; hence the rate of the initial oxidation is 0.9. The figures obtained by this calculation are shown in the last two columns of Table II. Pyruvate is the only substrate oxidized at a greatly reduced rate by strain A13. The reason why the initial rates for succinate and fumarate are also somewhat lower might be that adaptation in the mutant was less complete than in the normal strain, so that succinic dehydrogenase and fumarase were not built up to their highest levels. The surprisingly high oxidation rate of strain A13 for malate, which undergoes just one oxidative step before the break, is hard to reconcile with the rate for this compound in the normal strain. It does not seem likely that the mutant is actually able to oxidize malate at a faster rate. More probably the rate is close to the maximum possible for O_2 utilization on this substrate, and in the absence of competition for oxygen by lower products in the mutant, more is available for malate oxidation. Whatever the reason for this high rate may be, it is evident that pyruvate is the only substrate in this group of compounds whose oxidation is materially slower in the mutant than in the parent strain. The initial rates of attack on succinate and fumarate are similar in both strains.

Although substrates other than acetate, ethyl alcohol, and malonate do not support the growth of the mutant, it was of interest to find out whether the energy from the oxidation of such substrates was utilizable for growth of the organism when acetate was also provided. The acetate is oxidized in preference to the other substrate, but since a certain amount of the latter is also decomposed, growth would be expected to be somewhat heavier than on acetate alone. Media containing acetate and succinate were inoculated with cells already adapted to oxidize succinate. The amount of growth was determined after 24 hours. As shown in Table III, a definite increase in growth was caused by the succinate. Therefore the oxidation of succinate must in some way be useful to the organism, even though succinate cannot support growth by itself.

A series of experiments was done in an attempt to prove that pyruvate is oxidized by way of acetate in the normal strain. From what is known of the transformation of pyruvate in other microorganisms, it seems very likely that this is the main metabolic path of pyruvate in *Azotobacter*, and this interpretation is supported by adaptation experiments which have previously been described (2). A test for anaerobic decarboxylation of pyruvate by a manometric method was negative, showing that the initial attack on this substrate is indeed dependent on oxygen. An indication of a common pathway for pyruvate and acetate was obtained by a determination of the respiratory rate for each compound separately and for both combined. Succinate was added in one Warburg vessel to show that the maximum possible rate of oxidation had not been reached. Table IV shows that the rate on pyruvate and acetate together is no higher than that on acetate alone, as would be expected if the compounds are competing for the same enzymes. The significance of this experiment may be questioned, since the possibility that the rate of O_2 uptake may be limiting is not definitely excluded, because succinate does stimulate the rate of acetate oxidation under certain conditions.

TABLE III

Influence of Succinate on Growth of Strain A13 on Acetate

Substrate, 0.02 M	Turbidity at 24 hrs.	N_2 fixed
	$2 - \log G$	mg.
Acetate	0.13	0.92
Succinate	0.00	0.00
Acetate + succinate	0.16	1.36

TABLE IV

Separate and Combined Respiratory Rates for Lactate-Grown Strain A4.4, Adapted to Succinate

Substrate	Q_{O_2} (N) × 10^{-3}
Pyruvate	14
Acetate	16
Pyruvate + acetate	16
Succinate + "	21

An attempt was made to prove directly that pyruvate is oxidized via acetate by an isotope dilution method. Both pyruvate and C^{14}-labeled acetate were added to a culture. Unfortunately the acetate was decomposed preferentially when both substrates were present; thus the results were inconclusive. The theory that pyruvate is oxidized via acetate has therefore not been proved directly, but at least no evidence has been found against it.

Finally some experiments were carried out in an attempt to show that malonate is decomposed via acetate. According to Lineweaver (6) the first step is a decarboxylation to form acetate. However, his data are entirely inconclusive for two reasons: first, he used cells which were not adapted to malonate; second, the amount of carbon dioxide which he found to be split off before oxygen consumption started was too small to be measured by the Warburg technique. Obviously carbon dioxide liberation unassociated with oxygen uptake could not be determined by malonate-adapted cells under aerobic conditions, since such cells oxidize acetate readily. A manometric experiment was therefore done with malonate-grown cells suspended in a malonate medium under anaerobic conditions. Only a slight increase in pressure, amounting to approximately 10 per cent of the theoretical for complete decarboxylation to acetate, was observed and this soon ceased. Apparently the initial attack on malonate is dependent on oxygen. However, the rôle of oxygen in malonate decarboxylation is not clear. Oxygen might cause an oxidation of the malonate before it is decarboxylated or it might bring about an activation of the malonate, for example, by a phosphorylative mechanism.

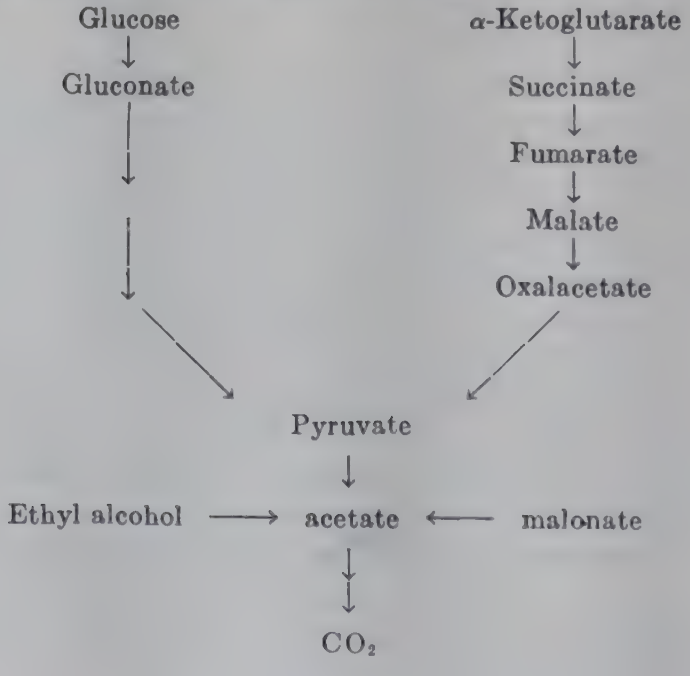
DISCUSSION

The experiments in this paper have shown that there is no reason to suspect anything abnormal in the metabolism of strain A13 except the rate of breakdown of pyruvate. From Table II it is evident that succinate, fumarate, and malate can be attacked at a rate comparable with that of the normal strain if adaptation to the first two substances is permitted to take place. This adaptation is also required by the normal strain. The over-all rate for these compounds is always slower in the mutant, as is to be expected, since the reaction is practically arrested when oxalacetate or pyruvate is reached.

All the anomalies of strain A13 can be accounted for if it is assumed that this organism has a metabolic block in the conversion of pyruvate to acetate. To explain the growth disturbances it has to be further postulated that acetate or some similar oxidation product is indispensable for growth of *A. agilis* and must be supplied either as such or in the form of substances which can be oxidized to acetate. Any compound which cannot be converted to acetate is then unable to support growth. This assumption is not an unreasonable one, since independent evidence has been obtained for the constant formation of acetate from other substrates in the normal strain (2), and since the experiments in this paper have shown that the mutant strain can oxidize higher substrates and obtain energy from them. It must be noted that acetate is required in large quantities, since most of it is rapidly decomposed to carbon dioxide and water. In the case of strain A13, acetate or an acetate substitute can still be formed from alcohol and malonate.

The fact that pyruvate is still attacked at a slow rate by the mutant strain has to be accounted for. The rate is only one-fortieth of the normal, as shown in Table II. No growth accompanies this oxidation; thus it is certainly not leading to the same products as oxidation of pyruvate by the normal strain. If acetate were formed, the organism would be expected to grow, since a mixture of pyruvate and acetate permits growth. The theory is therefore suggested that pyruvate is decomposed in the mutant by a slow, secondary mechanism which does not lead to acetate.

Certain conclusions concerning the pathways of substrate oxidation by *A. agilis* can be drawn from the behavior of the mutant strain. It would seem reasonable to assume that any compound that supports growth of the normal strain, but not of the mutant, is normally metabolized by way of pyruvate. A list of these substances has already been given. Evidence has been presented in a previous publication for the existence of a chain of reactions starting with α-ketoglutarate and going through succinate, fumarate, malate, oxalacetate, and pyruvate to acetate (2). On the other hand, unpublished experiments have shown that glucose-grown cells are unable to oxidize α-ketoglutarate, succinate, or fumarate until time has been permitted for adaptation to take place, while pyruvate is readily attacked without adaptation. This indicates that the hexose sugars are oxidized to pyruvate by a pathway not involving α-ketoglutarate. The actual mechanism leading from glucose to pyruvate is not known, but gluconic acid is a suspected intermediate, because growth on glucose leads to adaptation to gluconate, while the reverse is not the case. This was shown by Harris (7) and confirmed by me. On the basis of these considerations the accompanying scheme is suggested for the metabolic path-



ways in *A. agilis*. Acetate is oxidized to carbon dioxide by a completely obscure mechanism which does not seem to be related to the tricarboxylic acid cycle (2).

Another observation worthy of further mention is that adaptation rates are slow for the mutant when exposed to the substrates above the block, indicating that acetate or other oxidation products of pyruvate may be involved in enzyme synthesis. This prolongation of adaptation is not due to the absence of growth, since adaptation normally takes only a few minutes for certain substrates and must therefore be independent of growth.

Finally, it should be pointed out that the use of mutants for the study of catabolic reactions may be more limited than was thought at the outset. This is due to the adaptation difficulties. For instance, there was in the beginning no reason to suspect that strain A13 could not be used to demonstrate directly that glucose or any other complex substrate is metabolized via pyruvate. However, the strain can only be grown on acetate, alcohol, or malonate, and is therefore unadapted to other substrates. Adaptation to attack glucose at a sufficiently high rate could not be accomplished within the time limit since the mutant is not entirely stable. It had been expected that any mutant strain with a block in some major metabolic path might require two substrates for growth, one to supply the compounds above the block, the other the ones below. With strain A13 this was not true, possibly because the synthetic path differs from the oxidative one. But this would not always be expected to be the case, and a strain requiring two substrates for growth would naturally be adapted to both. Therefore, although the method has its limitations, it no doubt might prove to be a very important tool to eliminate one enzyme at a time from metabolic systems and cause accumulation of intermediate oxidation products.

SUMMARY

A mutant strain of *Azotobacter agilis*, characterized by inability to grow on any of the ordinary substrates except acetate, ethyl alcohol, and malonate, has been shown to be able to oxidize several more complex compounds to oxalacetate or pyruvate which accumulate in the medium. The energy derived from the oxidation of higher substrates is available to the organism, but growth is apparently impossible unless acetate or a similar compound either is supplied as such or can be formed during growth. All the abnormalities of the mutant can be explained by assuming that it is deficient with respect to an enzyme system catalyzing the oxidation of pyruvate to acetate. A scheme of oxidative pathways in *A. agilis* is suggested on the basis of previous and present evidence.

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AN IMPROVED MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF CYSTINE IN HUMAN URINE WITH LEUCONOSTOC MESENTEROIDES P-60*

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Investigations concerning the urinary excretion of cystine by normal subjects and by individuals with cystinuria, liver cirrhosis, diabetes, tuberculosis, and other pathological conditions have been reported previously from this laboratory (2–6). In continuing these studies, modification of the cystine assay method seemed desirable to minimize destruction of cystine which may occur in the medium as a consequence of the Maillard reaction (7–10). The authors' investigations led to the development of a microbiological procedure by which apparent browning of the medium was entirely eliminated. The response of *Leuconostoc mesenteroides* P-60 to L-cystine under the new conditions was approximately 10 times as sensitive as that obtained by the method employed previously in the authors' laboratory (2). Although closely agreeing cystine values for acid-hydrolyzed urine were obtained by the two procedures, those for untreated urine were much lower when determined under the improved conditions.

The objectives of the present report are (*a*) to describe the improved assay method as applied to the determination of cystine in urine and (*b*) to discuss the wide variation in values obtained under different assay conditions for cystine in unhydrolyzed urine.

EXPERIMENTAL

Both acid-hydrolyzed[1] and untreated urine samples were employed. Neutralized samples diluted 1:10 to 1:40 and standard L-cystine solutions were added to 13 × 100 mm. test-tubes in the manner described previously (12). Five dosage levels for each urine sample and fifteen standard L-cystine levels (0 to 1.4 γ per tube) were run in triplicate in each experiment. The Brewer automatic pipette was employed for each pipetting operation throughout the experiments.

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* Paper 65. For Paper 64, see Camien and Dunn (1). This work was aided by grants from the American Cancer Society through the Committee on Growth of the National Research Council. The authors are indebted to Katharine Remington Dunn, Samuel Eiduson, and Gene Molene for technical assistance.

[1] The hydrolysis procedure was essentially as described previously (11).

The basal medium was prepared as indicated in Table I. The amino acids of the medium were the same as in Medium D of Paper XVIII (14), except that cystine and hydroxyproline were omitted and the final concentration of the other amino acids was increased about 40 per cent. The other constituents of the medium were those given in Table I of Paper 29 (15), except that folic acid was substituted for the folic acid concentrate. The dry mixtures (Table I) were dissolved in distilled

TABLE I

Composition of Cystine Assay Medium

Mixture A		Mixture B	
	mg.		mg.
DL-Alanine	8181	Adenine sulfate·$2H_2O$	41.4
L-Arginine·HCl	327	Guanine·HCl·H_2O	39.0
L-Asparagine	1636	Uracil	36.0
L-Glutamic acid	614	Xanthine	36.0
Glycine	409	Sodium acetate	36,000
L-Histidine·HCl·H_2O	82	Ammonium chloride	12,000
DL-Isoleucine	614	KH_2PO_4	1,500
L-Leucine	307	K_2HPO_4	1,500
DL-Lysine·HCl	654	$MgSO_4\cdot7H_2O$	600
DL-Methionine	164	$FeSO_4\cdot7H_2O$	30
DL-Norleucine	409	$MnSO_4\cdot4H_2O$	30
DL-Norvaline	409	NaCl	1,050
DL-Phenylalanine	246	Thiamine·HCl	3.0
L-Proline	102	Pyridoxine	4.8
DL-Serine	327	Pyridoxamine·2HCl	0.30
DL-Threonine	1841	Pyridoxal·HCl	0.30
L-Tryptophan	41	Ca *dl*-pantothenate	6.0
L-Tyrosine	123	Riboflavin	6.0
DL-Valine	614	Nicotinic acid	6.0
		Biotin	0.015
		p-Aminobenzoic acid	0.30
		Folic acid	0.015
		Choline chloride	30.0
		Inositol	75.0

Amounts given are for 1000 assay tubes, each containing a final total of 3 ml. of solution. Mixture A and Mixture B were dehydrated and treated in a ball mill separately by the method of Camien and Dunn (13). Neither mixture was noticeably hygroscopic or harmed by prolonged storage in amberware bottles. 1.71 gm. of Mixture A and 5.16 gm. of Mixture B were dissolved in distilled water and diluted to 100 ml. to prepare medium for 100 assay tubes. 1 ml. of the solution (pH 6.32) was added to 1 ml. of diluted urine or L-cystine sample in each assay tube. The tubes were covered, sterilized in flowing steam at 100° for 30 minutes, and cooled. 1 ml. of sterile 6 per cent glucose solution was then added aseptically to each tube.

TABLE II

Recovery of Cystine from Amino Acid Test Mixture

Cystine in test mixture per tube	Titration volume*	Cystine found per tube	Cystine recovered	Deviation from mean value
γ	*ml.*	γ	*per cent*	*per cent*
0.188	5.71	0.182	96.7	2.1
0.375	7.08	0.372	99.1	0.3
0.563	8.08	0.546	97.1	1.7
0.750	9.28	0.768	102.3	3.5
0.938	10.00	0.930	99.2	0.4
Average................................			98.9	1.6

The mixture contained 2.4 per cent L-cystine in addition to the following percentages: DL-alanine 6.5, L-aspartic acid 9.7, L-arginine·HCl 3.9, L-glutamic acid 6.5, glycine 4.8, L-histidine·HCl·H_2O 4.4, DL-isoleucine 6.5, L-leucine 4.8, L-lysine·HCl 6.1, DL-methionine 3.2, DL-phenylalanine 4.8, L-proline 4.8, DL-serine 13.0 DL-threonine 9.7, L-tyrosine 2.4, and DL-valine 6.5. Three additional assays of the given mixture yielded recoveries of 100.2, 98.0, and 103.4 per cent with mean deviations from the means of 1.5, 0.8, and 3.1 per cent, respectively.

* Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.

TABLE III

Assay of Cystine in Untreated Urine

Improved method			Original method				
			Urine added per tube*	Autoclaved 20 min.		Autoclaved 40 min.	
Urine added per tube*	Titration volume†	Cystine found‡		Titration volume†	Cystine found‡	Titration volume†	Cystine found‡
ml.	*ml.*	*mg.*§	*ml.*	*ml.*	*mg.*	*ml.*	*mg.*
0.005	5.42	43.8	0.02	2.93	92.0	2.79	110
0.010	6.54	42.1	0.04	4.15	93.4	3.69	105
0.015	7.50	41.5	0.06	5.11	87.7	4.50	102
0.020	8.31	40.9	0.08	6.19	96.2	5.09	102
0.025	8.82	38.7	0.10	6.70	90.6	5.53	101
Average		41.4			92.0		104

* Amounts calculated in terms of undiluted sample.

† Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.

‡ Calculated in terms of excretion per 24 hours.

§ The downward "drift" of values which may be noted in this assay was not commonly observed with the improved method. The average value, 42.0, was found with two additional determinations (improved procedure) for the same sample.

water[2] as required and 1 ml. of the solution (pH 6.32) was added to 1 ml. of diluted urine or L-cystine sample in each test-tube. The tubes were covered, sterilized in flowing steam at 100° for 30 minutes, and cooled. 1 ml. of sterile 6 per cent glucose was then added aseptically to each tube. The tubes were autoclaved after adding the glucose in some of the experiments to obtain (for comparison) cystine assay conditions approximating those employed previously in this laboratory (2).

The methods for maintaining the *L. mesenteroides* P-60 culture, preparing the inoculum suspension, and inoculating the tubes have been described previously (12). It was found convenient in many of the experiments, however, to add inoculum suspension to the sterile 6 per cent glucose solution until the latter was barely turbid. Inoculation and addition of the glucose solution were then accomplished in a single pipetting operation.

TABLE IV

Assay of Cystine in Hydrolyzed Urine

Improved method			Original method				
				Autoclaved 20 min.		Autoclaved 40 min.	
Urine added per tube*	Titration volume†	Cystine found‡	Urine added per tube*	Titration volume†	Cystine found‡	Titration volume†	Cystine found‡
ml.	*ml.*	*mg.*	*ml.*	*ml.*	*mg.*	*ml.*	*mg.*
0.005			0.02	2.70	77.1	2.50	89.8
0.010	6.08	76.5	0.04	3.61	78.6	3.09	80.3
0.015	7.21	70.8	0.06	4.39	74.0	3.62	75.6
0.020	8.28	70.2	0.08	5.11	70.9	3.97	66.2
0.025	9.40	72.1	0.10	5.52	66.3	4.28	61.4
Average		72.4			73.4		74.7

* Amounts calculated in terms of undiluted sample.

† Calculated as ml. of 0.01 N NaOH to titrate 1 ml. of medium.

‡ Calculated in terms of excretion per 24 hours.

The inoculated tubes were incubated for 72 hours at 35° and then steamed for 20 minutes at 100° before titrating as described previously (12).

The experimental results are given in Figs. 1 and 2 and Tables II to IV. Samples (prepared by Mr. I. Porush in the authors' laboratory) of DL-cystine and D-cystine ($[\alpha]_D^{25}$ = +209° in 1.003 N HCl) had activities of 49.2 and 1.5 per cent, respectively, compared to analytically pure L-cystine, as determined by the authors' improved procedure.

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[2] The small amount of insoluble residue was removed by filtration or decantation.

DISCUSSION

L. mesenteroides P-60 was selected for the determination of cystine in urine, since it has been found to be more specific in its requirement for this amino acid than *Lactobacillus arabinosus* 17-5, *Lactobacillus fermenti* 36, *Lactobacillus casei*, *Lactobacillus pentosus* 124-2, *Lactobacillus delbrueckii* LD-3, *L. casei* (*delbrueckii*) LD-5, and *Streptococcus faecalis* R (10, 16).

Considerable evidence has appeared in recent literature (7–10), indicating that the Maillard reaction in microbiological assay media is accompanied by considerable destruction of nutrients, especially cystine. Although it has been suggested that separate sterilization of the glucose (7, 9) or substitution of sucrose for glucose (8, 9) will eliminate apparent browning in the medium, it does not appear that cystine in urine has previously been determined under conditions which minimize browning and cystine destruction.

Browning of the medium was avoided in the present experiments by separate sterilization of the glucose.[3] Under these conditions the amounts of cystine found in untreated 24 hour urine samples from five normal male subjects averaged[4] 48.8, 42.0, 34.8, 63.2, and 41.2 mg. respectively. The same samples yielded 64.9, 63.5, 59.7, 89.4, and 51.1 mg. after hydrolysis. That these values may be of relatively high accuracy was indicated by recoveries of cystine from an amino acid test mixture (Table II), averaging 100.1 per cent for four separate assays (98.8, 100.2, 98.0, and 103.4). The average deviation from the mean calculated for repeated assays of the same sample averaged 4.5 per cent for seventeen 24 hour samples (from normal and tuberculous[5] subjects) in which cystine was determined on two to four separate occasions, indicating that a satisfactory degree of reproducibility may be obtained by the described procedure. Mean deviations from the means for the five dosage levels in the individual assays averaged 4.3 per cent for a total of 56 assays run in a number of experiments.

The values found for cystine in normal untreated urines (except for the individual who excreted 63.2 mg. per 24 hours) were considerably lower than those reported in the literature (2, 4, 5, 18, 19). Since the values (given in the preceding paragraph) found for normal urine after hydrolysis were approximately the same as those reported previously (4), it seemed likely that the discrepancy in values obtained for untreated samples was due to a difference in the extent to which the free cystine in the standard (and hydrolyzed samples) and the bound cystine in the untreated samples were destroyed.

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[3] It is not likely that sucrose could have been substituted for glucose since this disaccharide does not appear to be utilized by *L. mesenteroides* P-60 (17).

[4] Each value is the average of two separate determinations.

[5] Data for the tuberculous subjects are to be published elsewhere.

The data given in Tables III and IV, obtained by determining cystine in a single urine sample before and after hydrolysis as well as under conditions enhancing and minimizing the Maillard reaction, appear to support this hypothesis. An untreated 24 hour urine sample yielded 41.4 mg. of cystine when assayed by the improved procedure, but 92.0 and 104 mg., respectively, were found for the same sample when the assay tubes containing glucose were sterilized in the autoclave for 20 and 40 minutes.[6] After hydrolysis, however, this sample yielded 73 ± 2 mg. under each of the three assay conditions. It may be noted from the standard curves obtained under these varying conditions (Figs. 1 and 2) that the sensitivity of response was increased approximately 10-fold with the improved pro-

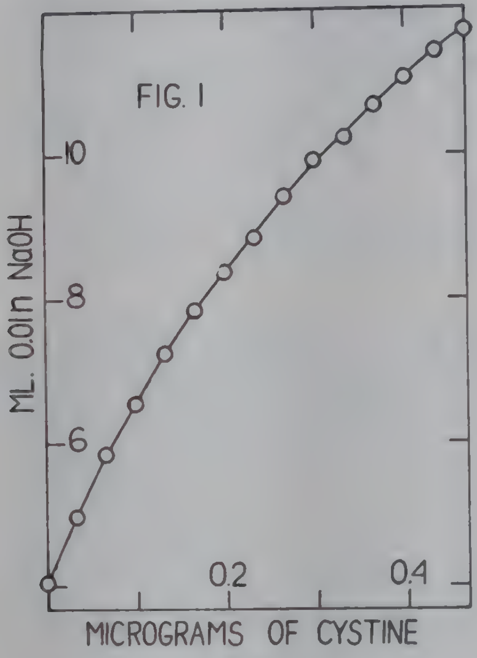

Fig. 1

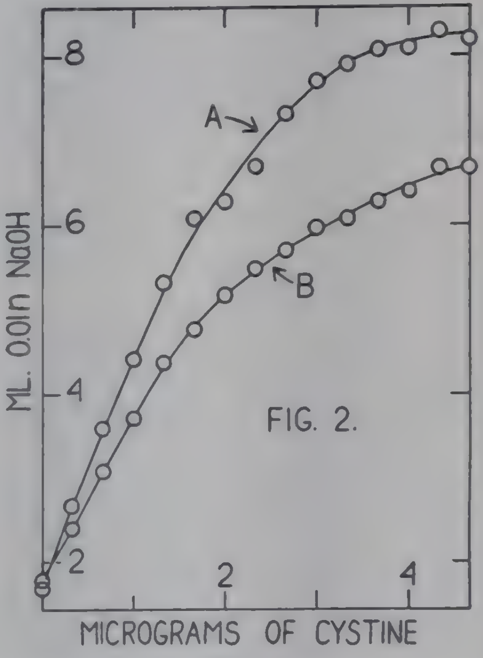

Fig. 2

Fig. 1. Response of *L. mesenteroides* P-60 to L-cystine. Glucose in sterile solution was added aseptically to the assay tubes after steam sterilization of the latter (containing no glucose before sterilization) at 100° for 30 minutes. The micrograms of cystine and the ml. of 0.01 N NaOH are the amounts calculated per ml. of final solution. Each point on the curve represents the average titration from three assay tubes (13 × 100 mm. tubes, each containing 3 ml. of final solution).

Fig. 2. Response of *L. mesenteroides* P-60 to L-cystine. The assay tubes contained glucose before sterilization and were autoclaved at 15 pounds for 20 minutes (Curve A) and 40 minutes (Curve B) respectively.

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[6] Urea or other substances present in the unhydrolyzed urines may inhibit the Maillard reaction sufficiently to explain in part these results.

cedure.[7] Destruction of nutrients other than cystine or production of toxic substances as a consequence of the browning reaction may be indicated by the lower maxima approached by Curves A and B in Fig. 2.

SUMMARY

An improved microbiological method for the determination of cystine in urine has been described. 24 hour urine specimens from five normal male subjects yielded 35 to 63 mg. of cystine before hydrolysis and 60 to 89 mg. after hydrolysis. The analytical results appeared to be of relatively high dependability, since an average of 100.1 per cent of the L-cystine in an amino acid test mixture was recovered and satisfactory agreement of results for cystine in urine samples was obtained at different dosage levels and with repeated determinations. Higher values (similar to those reported in the literature), obtained by the original procedure for cystine in untreated urine, were shown to result from unequal destruction of standard and sample cystine as a consequence of the Maillard reaction.

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[7] That the responses in Fig. 2 are as good as they are after 20 and 40 minutes autoclaving may probably be attributed to the relatively low initial pH (6.32) of the medium. The browning of the medium is much more extensive at higher pH values, and the lability of the cystine would be expected to increase with the pH.

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ANALYSIS FOR VITAMIN B_{12} AND VITAMIN B_{12a} BY PAPER STRIP CHROMATOGRAPHY

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Methods for separation of various growth factors, active for *Lactobacillus lactis* or *Lactobacillus leichmannii*, have been described. Chromatographic techniques employed by Lester Smith allowed separation of two red bands containing substances clinically active against pernicious anemia and having microbiological activity with *L. lactis* (1). Studies by Cuthbertson and Lester Smith with partition chromatography on paper, combined with microbiological assays on a solid medium, permitted demonstration of these two factors in concentrations of 0.005 to 0.1 γ. Water-saturated *n*-butanol was used as solvent with either upward or downward development (2).

A similar method, employed by Winsten and Eigen, showed double growth zones in the microbiological assay following partition chromatography of a concentrate of vitamin B_{12} and of a crystalline antipernicious anemia factor. Incomplete separation of the zones was obtained, the R_F values ranging between 0.0 to 0.03 and 0.03 to 0.10 for the two substances (3).

Additional substances which had growth-promoting activity for the assay organisms, but moved rapidly, were noted upon chromatographing liver extracts and animal protein factor concentrates. One of these substances had an R_F value characteristic of thymidine. Additional desoxyribosides, which have growth factor activity for *L. leichmannii* (4), may account for other zones found following the plating of a developed strip on a microbiological assay medium.

With the basic methods described above, we have been successful in defining conditions which allow quantitative separation of vitamins B_{12} and B_{12a} from mixtures of these two substances. The method has been applied with fermentation broths and liver concentrates for the separation of vitamin B_{12}- and B_{12a}-like materials from other microbiologically active growth factors.

Procedures

In our work, both ascending and descending methods of paper strip chromatography have been employed. The latter procedure is preferable, since extended diffusion times may be used to obtain complete separation. Whatman No. 1 filter paper was cut in strips 1 × $22\frac{1}{2}$ inches. Experiments have been made with untreated strips or with buffered strips, as indicated below. Strips were impregnated with various salts by passage through 0.66 M solutions, followed by drying in air. The vessels used for development were large cylinders $6\frac{1}{2}$ × 30 inches, closed at one end, or a rectangular vessel $9\frac{1}{2} \times 29\frac{1}{2} \times 28\frac{1}{2}$ inches. Absorbent cotton moistened with water was placed in the bottom of each vessel. A dish containing water-saturated organic solvents was placed beneath the strips and a trough filled with solvent was used for immersion of the upper ends of the strips. The vessels were covered with a glass plate and sealed with silicone. Water-saturated solvents were employed in all cases. Benzyl alcohol, cresol, *n*-butanol, and secondary butanol were used in the survey experiments. A temperature of about 28°, in an area with little air movement, was maintained except where noted.

Strips were spotted with 0.01 ml. of a water or a solvent solution of material containing vitamin B_{12}. When the concentration of vitamin B_{12} was inadequate, successive spottings were made, and a continuous flow of warm air used to promote rapid drying. The minimum concentration of vitamin B_{12} or B_{12a} detectable was about 0.002 γ. Since diffusion rates were somewhat variable from day to day, control samples of pure vitamin B_{12} were always included.

Microbiological fermentation broths were extracted by techniques described in the literature to yield concentrated samples, for ease in application to the paper strip. Either butanol extraction from an $(NH_4)_2SO_4$-saturated broth (5) or extraction with *o*-cresol (6) was successfully employed.

For the former extraction, 0.5 volume of water-saturated butanol was mixed with 1 volume of a butanol-saturated water solution of LLD factors.[1] Excess $(NH_4)_2SO_4$ was added, followed by thorough mixing. The extraction was repeated until microbiological assay showed no further decrease in the LLD factor content of the broth. A measured portion of the butanol layer was spotted for development. In the cresol extraction, repeated treatment with 0.1 volume of *o*-cresol served to extract the major portion of vitamins B_{12} and B_{12a}.

After spotting, strips were placed in the saturated atmosphere of the solvent vessel for equilibration for 2 hours before solvent was added to the trough to initiate diffusion down the strip. Following development of the chromatogram with the solvent, strips were laid on large rectangular assay plates containing the inoculated medium employed in the cup assay with *L. lactis* Dorner (7, 8). After incubation overnight, strips were removed from the agar, revealing oval zones of growth beneath the location of LLD-active substances. Duplicate strips were sectioned and leached into 10 ml. of water to yield solutions for quantitative assay by the *L. lactis* Dorner titrimetric procedure (9).

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[1] Fermentation broths contain a variety of substances which produce a vitamin B_{12}-like response in microbiological assays with *L. lactis* Dorner. These substances are called LLD factors (9).

EXPERIMENTAL

Plain paper strips were employed for preliminary experimentation with mixtures of vitamins B_{12} and B_{12a}. No movement from the point of application was produced by development with benzyl alcohol. Movement but no separation was obtained with secondary butanol. The toxic effect of cresol prevented demonstration of microbiologically active areas on paper strips, even after attempts to remove the cresol with ether. *n*-Butanol development showed a double zone, similar to that described by Winsten and Eigen (3). Thymidine could readily be distinguished with the latter solvent, since it moved rapidly along the strip with an R_F value of about 0.5.

TABLE I

Chromatographic Development of Vitamins B_{12} and B_{12a} on Impregnated Strips

Vitamin tested	Impregnating salt			
	Citric acid-Na citrate, pH 1.9	Citric acid-Na citrate, pH 3.0	KH_2PO_4, pH 4.6	NaCl
	Diffusion, mm. from origin			
B_{12a}	0	7	18	70
B_{12}	0	23	58	71

Attempts were made to promote separation of vitamins B_{12} and B_{12a} with *n*-butanol as a developing agent by impregnating strips with various salts at different pH levels (Table I). The time of development of strips with solvent was lengthened to 2 days to promote the separation. The best separation was obtained with strips impregnated with KH_2PO_4. No movement was obtained with plain paper strips or at pH 1.9. Although some separation was obtained at pH 3.0, the zones appeared as doublets. NaCl promoted diffusion but no separation.

More rapid separation was obtained with increased temperature. Strips impregnated with KH_2PO_4 showed LLD-active areas by microbiological assay for vitamin B_{12a} 45 mm. from the origin and for vitamin B_{12} 170 mm. from the origin following 45 hours development at 37°. This result may be compared with results recorded in Table I for development at 24° for a similar time period, or with a result of 57 mm. and 230 mm. at 24° for 72 hours.

Pure vitamin B_{12a}, obtained by hydrogenation of vitamin B_{12} (10), invariably produced a single well defined zone of growth under paper strips in the microbiological assay, as shown in Strip *b*, Fig. 1. Vitamin B_{12} likewise showed a single growth zone when concentrations lower than 0.01 γ were placed on the strips. At higher concentrations of B_{12}, however, a second zone, having light growth, appeared also in the vitamin B_{12a} position (Fig. 1, Strip *a*). With mixtures of vitamin B_{12a} and high

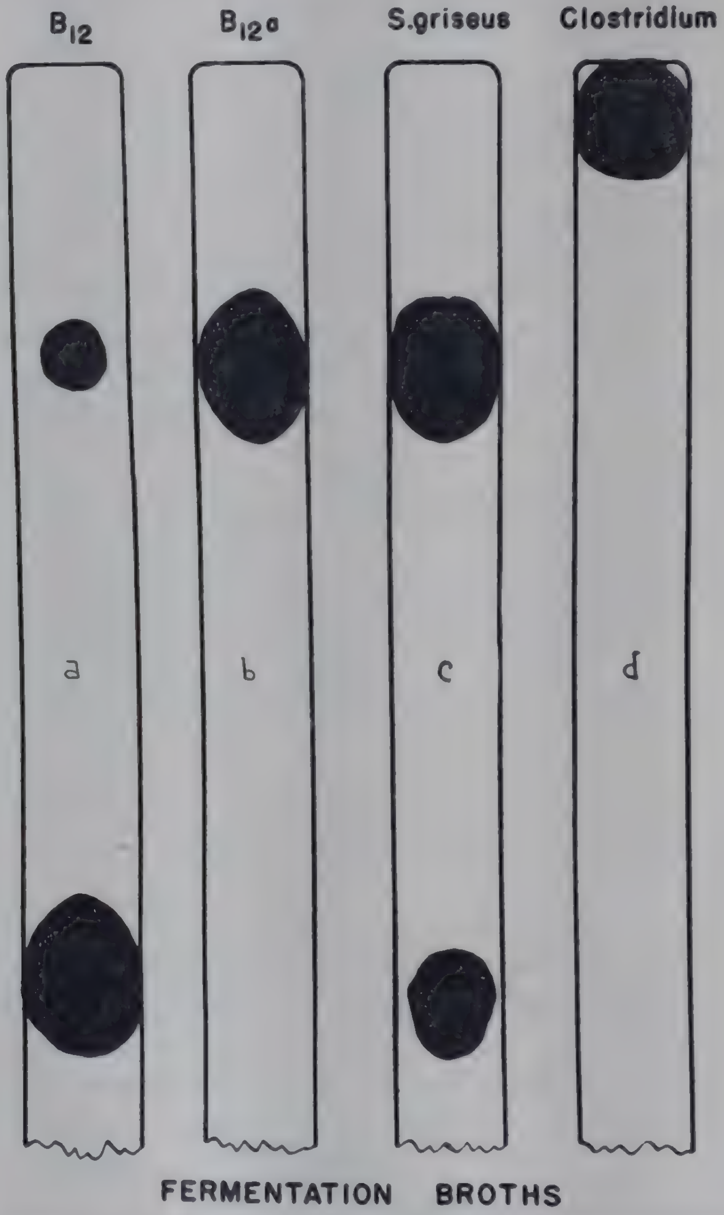

FIG. 1. Representative drawings of paper strip chromatographs. The inked spots are areas which promoted growth of *L. lactis*. Strips *a*, *b*, *c*, *d* are described in the text.

concentrations of vitamin B_{12}, the vitamin B_{12a} zone and the second zone derived from vitamin B_{12} were superimposed, indicating that the second zone probably was vitamin B_{12a}. Additional evidence was obtained by comparative microbiological assay of the leachings from a strip spotted with a very high concentration of vitamin B_{12} (Table II). As will be noted, a low microbiological assay value with *L. leichmannii*, characteristic of vitamin B_{12a} (11), was found for the first zone obtained from vitamin B_{12}.

Crystalline vitamin B_{12} of 95 per cent purity, as determined by solubility analysis,[2] was used in these studies. It contained no chemically detectable amount of vitamin B_{12a}. Evidence that conversion of vitamin B_{12} to B_{12a} took place during the chromatographic analysis, and was not present in the original sample, was obtained by rechromatographing the leachings from the above experiment. The slow moving zone rechromatographed into a single zone, which is characteristic of vitamin B_{12a}. The rapid moving zone rechromatographed into two zones, the major one being vitamin B_{12} and the minor one characteristic of vitamin B_{12a}. Leaching of the rechromatographed zones, followed by a third chromatographing, gave similar results.

TABLE II

Comparative Microbiological Assay of Two Zones Derived from Vitamin B_{12} by Paper Strip Chromatography

Sample	Distance from origin	Vitamin B_{12} activity	
		L. lactis assay	*L. leichmannii* assay
	mm.	γ	γ
Zone 1	43	1.1	0.41
" 2	196	15.2	13.0
Crystalline vitamin B_{12a}, per gamma		0.9	0.2
Crystalline vitamin B_{12}, per gamma		1.0	1.0

Over the normal range of working conditions of the method, a fairly constant proportion of vitamin B_{12a} is obtained from vitamin B_{12}. It has been our practice to assume a correction factor based on conversion of 10 per cent of the observed vitamin B_{12} to B_{12a} during the chromatographic procedure, since approximately this degree of conversion was obtained in control experiments. Table III presents observed vitamin B_{12a} percentages obtained from chromatography of various concentrations of vitamin

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[2] We wish to thank Mr. F. A. Bacher for solubility analysis and solvent distribution studies on the vitamin B_{12} preparation.

B_{12} and presents recovery data which demonstrate the quantitative nature of the procedure.

The procedure has been used for estimation of the concentration of vitamins B_{12}- and B_{12a}-like materials produced by microorganisms. Adequate concentrations of activity for spotting were obtained by solvent extraction procedures. The assay has also been applied to concentrates obtained from fermentation sources, which have antipernicious anemia activity, and to U. S. P. liver concentrates. These substances contain sufficient activity so that they may be applied directly to the paper strip without preliminary solvent extraction. Examples of the results of assays are presented in Table IV.

TABLE III

Quantitative Microbiological Assay of B_{12a} Zone from Chromatography of Various Quantities of Vitamin B_{12}

Vitamin B_{12} chromatographed	Vitamin B_{12} equivalent of zones		Per cent of vitamin B_{12} chromatographed found in vitamin B_{12a} zone
	Vitamin B_{12a} zone	Vitamin B_{12} zone	
γ	γ	γ	
91	7.9	84	8.7
68	7.0	55	10.3
45	5.0	37	11.1
22	2.6	21	11.8

TABLE IV

Analysis of Fermentation Broths and Antipernicious Anemia Concentrate by Paper Strip Chromatography

Culture	Nitrogen source	Fractionation of microbiological activity*		
		Solvent soluble	Vitamin B_{12} zone	Vitamin B_{12} zone
S. griseus strain 1	Whole protein medium	51	18	1.9
" " " 1	Protein hydrolysate medium	69	34	<0.4
" " " 1	Synthetic medium	70	<2	15
" " " 2	Protein hydrolysate medium	80	24	1.5
Streptomyces sp.	" " "	67	20	<0.4
Cl. tetanomorphum	Brain-heart medium	53	<2	<2
	15 U. S. P. unit liver extract	†	97	<3.0

* The figures represent the per cent of *L. lactis* Dorner activity of the original sample recovered in various fractions.

† Sample spotted directly on strips without solvent extraction.

There was great variation in the proportion of total vitamin B_{12} to

B_{12a} type of compounds found in different fermentation broths by paper strip chromatography in relation to the microbiological LLD activity of the broths. Compounds having R_F values similar to vitamin B_{12} or to vitamin B_{12a} are present in some fermentation broths, but these compounds may represent nearly all or less than 10 per cent of the microbiologically active growth factors for *L. lactis* contained in the broth. The culture medium and the microorganism both are influential in determining the concentration and proportion of the growth factors isolated. Table IV shows evidence of the range of results obtained from the growth of a single strain of *Streptomyces griseus* on three media. Also, variation is noted among several cultures. Fig. 1, Strip *c*, represents the indicator strip obtained with fermentation broth of *S. griseus* (strain 2) grown on a protein hydrolysate medium. LLD titrimetric assay showed 1.5 per cent of the original broth activity to be in the vitamin B_{12} zone and 24 per cent in the vitamin B_{12a} zone.

Thymidine and related compounds which have LLD activity are present in fermentation broths, but they are leached off the paper by chromatographic technique. An additional microbiologically active compound which is retained on the paper strip is present in some fermentation broths. Fig. 1, Strip *d*, represents the chromatographic analysis of solvent-extractable material produced by a species of *Clostridium*. The quantitative data obtained with this culture are presented in Table IV. Less than 2 per cent of the LLD activity of the broth was found to have R_F values similar to those for vitamin B_{12} or B_{12a}, but approximately half the LLD activity was retained at the point of application on the paper strip. Solvent concentrates from this culture, containing the non-moving microbiologically active factor, yielded negative results when tested for vitamin B_{12}-like growth-promoting activity in chicks. With some cultures the non-moving zone and the vitamin B_{12a} zone form a doublet, making it impossible to obtain a quantitative evaluation for the vitamin B_{12a} type of material.

A liver extract for parenteral use in pernicious anemia (15 U. S. P. units per ml.) was found to contain activity equivalent to 17 γ of vitamin B_{12} per ml. by titrimetric assay with *L. lactis*. However, on paper strip analysis no vitamin B_{12} was present, but activity equivalent to 16.5 γ of vitamin B_{12a} was contained in the vitamin B_{12a} area of the paper strip. The indicator strip for this sample was indistinguishable from that represented in Fig. 1, Strip *b*, for vitamin B_{12a}.

SUMMARY

A method has been presented for quantitative estimation of vitamins B_{12} and B_{12a}, combining microbiological assay and paper strip chromatog-

raphy. A small corrective factor is required to compensate for conversion of vitamin B_{12} to vitamin B_{12a}, which may take place during the analysis. The method has been applied to microbiological fermentation broths and to injectable liver extracts (U. S. P.) for the analysis for vitamin B_{12} and B_{12a} type of materials. No quantitative relationship was found between the amount of these vitamins contained in the samples and the *Lactobacillus lactis* titrimetric assay of the samples.

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ALTERATIONS IN BLOOD GLUCOSE FOLLOWING INTRAVENOUS GALACTOSE

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No uniformity of response is evident in the reports of blood glucose concentrations observed following the administration of galactose. Oral administration of the latter sugar has been reported to elevate (1–4), depress (5–7), or not alter the blood glucose levels of healthy adults (8, 9), to increase that of subjects with diabetes mellitus (5, 10, 11), and to decrease greatly that of infants with idiopathic intolerance to galactose (12–17). The few studies with healthy infants indicate little or no change in glucosemia (12, 15). Finally no data have been found on the response of the blood glucose to galactose following its *intravenous* injection in infants or in adults with decreased utilization of the latter sugar.

To clarify the relationship between galactose and glucose the former sugar has been administered intravenously, in amounts usually employed in galactose tolerance tests, to thirteen healthy adults, five convalescent infants, four subjects with diabetes mellitus, thirteen adults with impaired utilization of galactose, and to one infant with idiopathic galactose intolerance. Since a major object of the study was to determine accurately the rate of utilization of galactose as well as alterations of blood glucose, the methods of analysis and calculation were carefully defined in control studies. As a result, a possible explanation for the differences in previous reports of changes in blood glucose has appeared.

Subjects and Methods

The subjects ranged in age from 8 weeks to 57 years. Activity was limited during the test. Galactose was injected intravenously as a 50 per cent solution in water in 3 to 7 minutes during the postabsorptive state. The infants received 1.2 gm. of the sugar per kilo of body weight; the others were given 0.4 to 0.6 gm. per kilo of body weight. Insulin was withheld the morning of the test from the patients with diabetes mellitus. None of them had acetonuria. The procedure was modified in seven of the healthy adults by the oral administration of 0.3 ml. of 95 per cent ethyl alcohol per kilo of body weight, 15 minutes before injection of the sugar (18). This resulted in impairment of their ability to utilize galactose. In addition one infant with idiopathic intolerance to this sugar received alcohol in two studies in doses of 0.4 and 0.5 ml. per kilo of body weight 15 minutes before injection of galactose, and on one other occasion crystalline insulin, 2 units subcutaneously. Further information concerning these patients and their galactose metabolism has been previously presented (18, 19).

Venous blood was collected in bottles containing oxalate immediately before the injection and then at 15, 45, and 75 minutes after its mid-point. Two analytical procedures were employed; Somogyi's iodometric titration method for galactose because of its accuracy in the low concentrations encountered (20), and Benedict's method for total sugar because of its speed, simplicity, and reliability in the ranges analyzed (21). Galactose alone was not measured by Benedict's method, since the procedure is not dependable at concentrations below 40 mg. per cent, and consequently the 15 minute sample of blood would have been the only one in which the values of this sugar would have been consistently in the range for accurate measurement.

The samples for galactose determination were fermented with 6.0 ml. of 20 per cent bakers' yeast for ½ hour at room temperature. Control studies established the adequacy of these conditions to remove glucose without altering galactose. Prior to its use the yeast was washed six times with distilled water, and the hematocrit of each yeast suspension determined. Yeast blanks were found to be identical with water blanks when analyzed by the Somogyi procedure. Following the precipitation of protein from the glucose-free blood by zinc sulfate and sodium hydroxide (22), the filtrate was analyzed in duplicate for galactose. A reaction time of 25 minutes with the alkaline copper reagent was employed. 97 to 100 per cent of this sugar was recovered from known solutions. The blood specimen obtained immediately before the administration of galactose served as a blank for non-glucose-reducing substances remaining after fermentation, and was taken into account in all calculations of galactose values (18). All dilution factors, including the addition of the yeast (corrected for its hematocrit), were considered in the final calculation of the galactose levels.

The second 2.0 ml. aliquot of blood was not fermented, but was analyzed in duplicate for total sugar (glucose plus galactose) with a Somogyi filtrate (22). In most of these determinations the sugars were permitted to react with the Benedict alkaline copper reagent for 5 minutes. Glucose plus galactose was thus measured together, but galactose had only 65 per cent of the reducing power of glucose under these conditions. Further analysis of the relationship between reaction time and recovery of galactose revealed that the reducing power of galactose increased to 80 per cent of that of glucose, when the time ranged from 10 to 15 minutes. Glucose reduction was complete during all of these intervals. The findings are presented in Table I. They are consistent with the principles concerning copper reduction methods for sugar analyses described by Somogyi (20). It is clear that the time of reaction with the alkaline copper reagent must be carefully controlled if reproducible recoveries of galactose are to be obtained with a 5 minute period. The twenty-six experiments listed in the first part of Table I illustrate the accuracy with which this interval may be employed.

TABLE I

Recovery of Galactose by Benedict Sugar Method; 100 Mg. Per Cent Glucose As Standard and Varying Time of Reaction with Alkaline Copper Reagent

Total sugar, galactose + glucose		No. of experiments	Total sugar recovered	Galactose*	Galactose recovered
Reaction time	*mg. per cent*				
min.			*mg. per cent*	*mg. per cent*	*per cent*
5	50 + 100	2	133	33	66
	75 + 100	2	148	48	64
	100 + 100	3	162	62	62
	150 + 100	3	198	98	65
	200 + 100	3	230	130	65
	100 + 0	4	68	68	68
	150 + 0	5	98	98	65
	200 + 0	4	131	131	65
10	100 + 0	3	80	80	80
15	100 + 0	3	80	80	80

* Galactose calculated as total sugar minus glucose.

Calculation of Blood Glucose—It is evident that blood glucose concentrations were not measured directly but had to be calculated from the total sugar value and the galactose concentration in the following manner.

Glucose = total reducing capacity − 65 % galactose concentration

For example:

Total reducing capacity (Benedict method, 5 min. reaction time) = 204 mg. %
Galactose (Somogyi method) = 147 mg. %
Glucose = 204 − (0.65 × 147) = 108 mg. %

If Benedict's method with *glucose as the standard* were employed for measurement of *both "galactose alone" and "total sugar"* with a reaction time of 10 minutes, the following calculations would be necessary:

Total sugar (as glucose) = 140 mg. %
Residual reducing power (as glucose, following fermentation by yeast) = 80 mg. %
Actual galactose concentration = 80/0.8 = 100 mg. %
Glucose = 140 − 80 = 60 mg. %
but *not* 140 − 100 = 40 mg. %

In certain ranges this erroneous calculation will either mask elevations of blood glucose or indicate large declines in this sugar when none has occurred. The general application of this concept to other sugar methods such as those of Somogyi, Hagedorn-Jensen, and Folin-Wu, in which galactose and glucose differ from each other in reducing power, is clear (20, 23, 24).

Results

Carbohydrate concentrations of the blood of the subjects with normal galactose tolerance tests are presented in Fig. 1 and Table II. The healthy adults, with one possible exception (L. G.), had significant elevations of blood glucose, *i.e.* 10 per cent or greater, which were usually evident at 15 minutes. In one instance (J. R.) the maximum elevation was not observed for 45 minutes, and in one other (J. P.) until 75 minutes. Similar rises occurred in the diabetics and the convalescent infants. The increase was greatest in the infants, presumably as the result of the large dose of galactose. Following this elevation, glucose concentrations frequently fell to levels below the initial ones, except in the diabetic patients.

The infant with the greatest inability to utilize galactose had no increase in blood glucose on three occasions, although blood levels did not fall below 50 mg. per cent following galactose alone (Fig. 1, Table III). When he received alcohol or insulin beforehand, however, hypoglucosemia with its attendant symptoms appeared despite an elevated total sugar level. In contrast to J. S., the subjects with galactose tolerances impaired by the administration of alcohol did not develop hypoglucosemia. None of their glucose values differed significantly from those of the healthy adults without alcohol; although the response was more variable (Fig. 1, Table III).

The pattern of alteration of blood glucose in the subjects with hepatic disease differed from that of the healthy adults, since elevations were not evident at the 15 minute point, but were delayed (Fig. 1, Table III).

DISCUSSION

Blood glucose alterations following the rapid intravenous injection of galactose are related to the subject's ability to utilize, convert, store, or excrete the latter sugar. The relationship is complex, however, since the disappearance of galactose is a function of several processes, the rates of each of which presumably vary with time; furthermore, changes in blood glucose must be the result of the rates of formation and utilization of glucose. Elevations of blood glucose concentrations, therefore, have appeared when the rate of entry of glucose into the blood stream and body fluids exceeded its rate of removal from these compartments. Galactose has entered this sequence through its conversion into glycogen and glucose in the liver (25–29). Confirmatory evidence of the rôle of glucose utilization in this mechanism has appeared in reports that serum inorganic phosphorous levels fall (30), the R. Q. rises (31, 32), and lactic acid production increases (33) following administration of galactose to

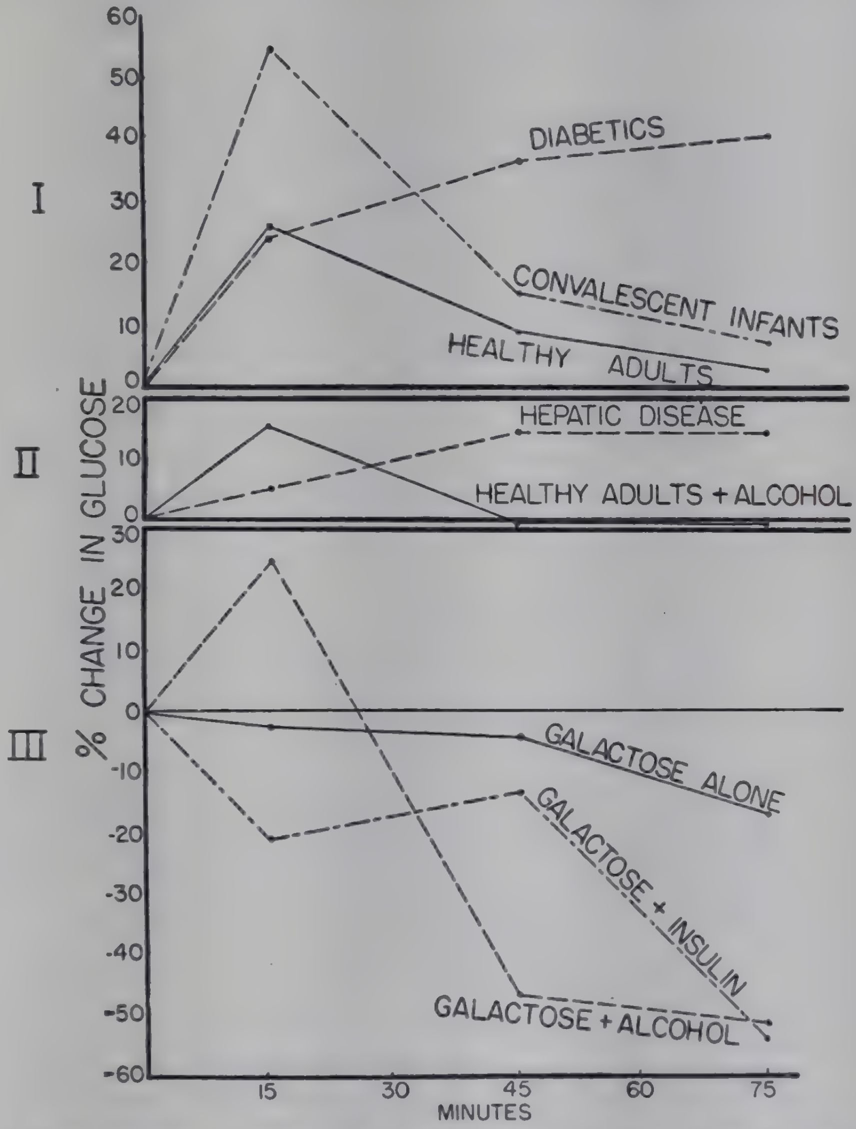

FIG. 1. Mean per cent changes in blood glucose. Section I, patients with normal galactose tolerance tests; Section II, adults with impaired ability to utilize galactose; and Section III, infant with idiopathic galactose intolerance.

TABLE II

Total Sugar and Glucose Response of Subjects; Normal Intravenous Galactose Tolerance Tests

Patient	Sex	Age	Weight	Galactose administered per kilo	Total sugar				Galactose			Glucose		
					0 min.	15 min.	45 min.	75 min.	15 min.	45 min.	75 min.	15 min.	45 min.	75 min.
Healthy adults														
		yrs.	*kg.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
B. M.*	M.	33	91	0.6	86	274	155	115	218	103	27	132	88	97
J. R.	"	33	70	0.6	80	179	120	94	150	30	10	81	100	86
L. G.†	"	27	68	0.6	88	171	100	84	125	27	12	90	82	76
J. P.	"	31	66	0.6	80	200	140	104	171	76	10	89	91	97
J. M.	F.	19	57	0.6	75	230	117	86	209	72	12	94	70	78
B. K. (*a*)	"	22	60	0.6	87	238	139	101	191	74	18	114	91	89
" " (*b*)	"	22	60	0.4	85	150	99	90	86	18	6	94	87	86
F. M.‡	M.	27	63	0.5	76	234	153	96	152	50	16	112	113	83
J. T. (*a*)	"	31	77	0.5	79	204	128	96	147	56	16	108	92	86
" " (*b*)	"	31	77	0.5	84	209	118	94	155	53	15	108	84	84
H. W.	F.	12	44	0.5	85	177	108	87	117	24	7	101	92	82
D. G.	"	23	52	0.5	86	188	95	79	131	7	3	103	90	77
I. L.	"	24	54	0.5	73	193		88	150		19	96		76
L. F.	"	35	50	0.5	72	182		84	120		12	104		76
M. C.	"	27	50	0.5	82	209	123	88	167	57	11	100	86	81
Diabetes mellitus														
M. S.	F.	15	43	0.5	66	158	120	119	95	12	3	96	112	117
M. B.	"	14	42	0.5	222	339	281	287	125	35	18	258	258	275
T. V.‡	M.	13	44	0.5	264	349	305	306	83	18	0	283	291	306
E. O.‡	"	14	53	0.5	141	276	229	209	108	25	5	190	209	205
Convalescent infants														
		wks.												
D. C.	F.	8	5.0	1.2	80	254	144	94	157	23	13	152	129	86
R. D.	M.	8	5.3	1.2	66	228	110	82	204	50	12	95	78	74
J. F.	"	16	3.7	1.2	73	230	92	88	212	48	8	92	61	83
C. Q.	"	18	4.4	1.2	62	246	108	72	205	64	15	113	66	62
G. H.	F.	28	5.4	1.2	76	216	134	88	174	78	15	103	83	78

* Received 54.6 gm. of galactose.

† 30 minutes total sugar = 133 mg. per cent; glucose = 95.

‡ Factor for conversion of galactose into its glucose equivalent = 0.8 in these experiments.

subjects with normal ability to utilize glucose, while these changes are not observed in diabetic subjects (34, 35).[1] In addition the temporary

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[1] Greenman, L., unpublished data.

TABLE III

Total Sugar and Glucose Response of Subjects; Impaired Intravenous Galactose Tolerance Tests

Patient	Sex	Age	Weight	Galactose administered per kilo	Total sugar				Galactose			Glucose		
					0 min.	15 min.	45 min.	75 min.	15 min.	45 min.	75 min.	15 min.	45 min.	75 min.
Healthy adults following alcohol														
		yrs.	*kg.*	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
J. R.	M.	33	70	0.6	97	242	173	145	195	111	59	115	101	107
L. G.	"	27	68	0.6	82	181	133	117	152	94	65	82	72	75
J. M.	F.	19	57	0.6	80	219	152	123	184	134	85	99	65	68
B. K.	"	22	60	0.5	81	202	154	122	182	121	82	84	75	69
J. T.	M.	31	77	0.5	81	192	156	137	179	106	74	76	87	89
I. L.	F.	24	54	0.5	81	180		124	156		68	79		80
M. C.	"	27	50	0.5	93	234		107	163		54	128		72
Hepatic disease; galactose alone														
J. C.*	M.	50	66	0.5	73	164	140	112	144	84	42	70	85	85
L. B.*	"	48	78	0.5	124	240		200	159		88	137		143
M. T.*	F.	57	48	0.5	87	172		103	132		23	86		88
W. P.†	M.	54	76	0.5	72	224	149	112	206	88	52	90	92	78
D. H.‡	"‖	53	67	0.5	80	209	161	139	151	85	25	88	93	119
E. K.§	F.‖	50	48	0.5	75	160	121	98	111	53	23	71	79	80
Infant with idiopathic galactose intolerance														
		wks.												
J. S. (galactose alone)	M.	21	5.0	1.2	72	207	187	151	222	170	155	63	76	50
		24	5.3	1.2	70	224	178	166	233	180	157	73	61	64
		27	5.5	1.2	77	255	207	190	272	208	188	78	72	68
J. S. (galactose + alcohol)	M.	29	6.0	1.2	67	234	146	130	218	188	168	92	24	21
		41	8.1	1.2	70	250	178	154	234	202	169	98	47	44
J. S. (galactose + insulin)	M.	26	5.6	1.2	48	164	132	100	194	138	122	38	42	21

* Cirrhosis of liver.

† Amyloidosis.

‡ Convalescent from homologous serum jaundice.

§ Biliary cirrhosis.

‖ Factor for conversion of galactose into its glucose equivalent = 0.8 in these experiments.

increase in blood glucose levels following galactose administration may be prevented by accelerating glucose utilization with insulin.[1]

Similar changes of blood glucose in the healthy adults in the experiments with, and those without, alcohol, despite significantly different galactose tolerance tests, cannot be definitely explained, although they seem to have been the result of the time interval required for alcohol to impair galactose utilization. In addition, no satisfactory explanation is apparent for the severe hypoglucosemia observed in the infant with idiopathic galactosuria when alcohol was administered with the galactose, especially since neither alcohol alone nor glucose alone evoked such a response.

Despite the variety of changes in glucosemia which may appear following the administration of galactose alone, it is not likely that profound decreases in blood glucose levels will occur. Reports indicating this occurrence must be reexamined (5–7, 12, 14–17), especially since the absence of symptoms of hypoglucosemia in infants with idiopathic intolerance to galactose has been ascribed to simultaneously elevated total blood sugars (glucose plus galactose). This observation has provided strong support for the theory that symptoms of hypoglucosemia do not necessarily depend on low blood glucose values, but rather on the level of total blood sugar. The explanation for these findings apparently lies in the failure to account for the different reducing powers of both sugars (12, 14–17). Recalculation of the data in these infants may explain their anomalous condition, since blood glucose levels then appear higher. This interpretation is confirmed by observations that galactose will not alleviate a severe insulin reaction despite the presence of an increased total blood sugar (5, 9), and that the infant with galactosuria presented here as well as the one described by Norman and Fashena (17) demonstrated symptoms of hypoglucosemia even though the total sugar was elevated.

SUMMARY

Alterations of blood glucose following the rapid intravenous injection of galactose are related to the subject's ability to metabolize the latter sugar, and depend on the resultant of the rate of entry of glucose into the blood stream and body fluids and its rate of removal from these compartments. The different reducing powers of glucose and galactose must enter into any calculation of the amounts of these two sugars, when they are present simultaneously in the blood stream. Although blood glucose values may rise, remain unchanged, or fall slightly following galactose administration, it seems unlikely that galactose alone will produce severe hypoglucosemia.

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ON THE ACTIVITY OF OXYBIOTIN*

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Recent advances in our knowledge of the metabolic activities of biotin (1–6) have increased interest in its biologically active analogue, oxybiotin. It is known that oxybiotin has biotin-like activity for several species of bacteria (7–10), yeast (7), and rats and chickens (11). In those cases which have been adequately tested (9, 12–14), it has been clearly demonstrated that oxybiotin is active *per se* and is not converted to biotin. In fact it has recently been suggested that oxybiotin might have biotin-like activity for all biological forms (15). However, since most of the early biological tests on oxybiotin were carried out before the multiple activities of biotin were known, it appears that the capacity of oxybiotin to replace biotin in every case cannot be inferred from its ability to carry out some of the functions of biotin. Two of the better known activities of biotin are its capacity to replace the need for oleic acid (16–18) and aspartic acid (1, 18, 19) for certain lactic acid bacteria. When biotin replaces aspartic acid, it causes aspartic acid synthesis (19), and it has been suggested that the mechanism involves carbon dioxide fixation (2, 3, 18, 20).

It is well known that oxybiotin is as active or nearly as active as biotin for a number of microorganisms in the presence of aspartic acid (7–10, 21–23). However, Stokes *et al.* (19) have indicated that the ability of oxybiotin to replace biotin in the absence of aspartic acid is negligible in the case of *Lactobacillus casei* and is only 0.2 to 1.0 per cent as great as biotin in the case of *Lactobacillus arabinosus* 17-5. For both of these organisms, oxybiotin shows a very high activity in the presence of aspartic acid. This brief report is presented to show that small changes in the initial pH of the bacterial medium will cause the apparent activity of oxybiotin in the absence of aspartic acid to vary several hundred fold and that these changes will mean the difference between poor and excellent growth during the usual type of microbiological test.

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EXPERIMENTAL

Methods and Materials

The basal medium consisted of glucose, acetate, amino acids, salts, purines, pyrimidines, and water-soluble vitamins (18). In order to obtain growth, the medium must be supplemented with either a small amount of biotin (0.5 mμgm. per 10 ml. of culture) and aspartic acid or a relatively large amount of biotin (20 mμgm.). The culture medium consists of 5 ml. of the basal medium supplemented as described and diluted to 10 ml. with distilled water. The assays were carried out in large size Evelyn colorimeter tubes plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure. The inoculum was a dilute washed saline suspension of a 12 to 24 hour culture of *L. arabinosus* 17-5. The growth was measured as turbidity (per cent transmission) with a tube of sterile culture medium as the blank or by titrating the acid produced with 0.1 N sodium hydroxide. The pH of the bacterial medium, a primary consideration in these experiments, was measured with a Beckman pH meter in preference to the less accurate visual methods. The term, initial pH, refers to the pH of the medium after autoclaving. The pH of the medium will decrease 0.1 to 0.2 upon autoclaving, if the pH is about 6.5, and somewhat more if it is above 7.0.

Results

It has been shown previously that in the presence of aspartic acid oxybiotin is 100 per cent (7, 21, 23) or 58 to 88 per cent (22) as active[1] as biotin for *L. arabinosus*. The results of a typical experiment are shown in Table I. While the activity of the two compounds did not agree as closely as this in every experiment, the actual differences were small and did not appear to be related to the initial pH of the medium.

In contrast to these results the growth response of *L. arabinosus* to biotin and oxybiotin at pH 6.5 in the absence of aspartic acid is shown in Table II. Here the biotin requirement is greatly increased (18, 19) and the initiation of growth is delayed (19). For the organisms receiving biotin maximum growth is attained in 38 hours. For the organisms supplemented with oxybiotin the onset of growth is greatly retarded and only mediocre growth is attained in 66 hours. The best growth is only about one-fifth of the maximum and oxybiotin does not appear to be the limiting factor since 2000 mμgm. give no better growth than 20 mμgm. These results are perhaps comparable to those of Stokes, Larsen, and Gunness (19) which indicated that 100 to 500 times as much oxybiotin is necessary in the absence of aspartic acid. Their medium was adjusted to pH 6.8 before autoclaving.

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[1] All expressions of activity and amounts of oxybiotin in this paper are made on the basis of the *d* isomer of the *dl* compound.

TABLE I

Comparison of Biotin and Oxybiotin in Presence of Aspartic Acid, Initial pH 6.6

	Biotin		Oxybiotin	
	Turbidity* at 24 hrs.	0.1 N acid at 66 hrs.	Turbidity at 24 hrs.	0.1 N acid at 66 hrs.
mμgm. per 10 ml.		*ml.*		*ml.*
0	85	4.0	85	4.0
0.125	76	8.7	77	8.6
0.25	71	10.2	72	10.0
0.375	63	11.9	66	11.8
0.5	61	13.0	61	12.9
0.625	58	13.9	58	13.9
0.75	55	14.6	55	14.6
1.0	50	15.5	50	15.4
1.25	46	17.1	48	17.0

* Per cent transmission in the Evelyn colorimeter. A reading of 100 indicates absence of growth.

TABLE II

Activity of Biotin and Oxybiotin in Absence of Aspartic Acid, Initial pH 6.5

Biotin					Oxybiotin				
	Turbidity*			0.1 N acid produced at 66 hrs.		Turbidity*			0.1 N acid produced at 66 hrs.
	24 hrs.	38 hrs.	66 hrs.			24 hrs.	38 hrs.	66 hrs.	
mμgm. per 10 ml.				*ml.*	*mμgm. per 10 ml.*				*ml.*
0	99	98	98	1.0	0	99	99	98	0.7
0.5	97	96	96	1.3	0.5	97	95	94	1.1
1.0	95	93	93	1.6	1.0	99	96	93	1.2
2.5	92	87	87	2.7	5.0	98	94	91	1.7
5.0	87	74	74	4.6	20.0	98	96	83	2.4
10.0	86	55	54	10.0	250.0	99	96	82	2.4
15.0	84	44	42	13.3	2,000.0	98	93	82	2.9
20.0	86	36	32	14.6	20,000.0	100	99	98	0.7

* See foot-note to Table I.

Table III presents the results of an experiment in which the initial pH was 7.0. It is evident that a more alkaline medium greatly favors the early growth of organisms supplemented with biotin and that the prolonged lag period previously recorded (19) for organisms synthesizing their own aspartic acid is eliminated. For organisms receiving oxybiotin, the lag period is considerably reduced but not eliminated. While growth is negligible at 24 hours, it is good at 38 hours and excellent growth has been obtained with moderate amounts of oxybiotin at 66 hours. By comparing the turbidity of the organisms at 66 hours, it is apparent that the response to 10 mμgm. of oxybiotin falls between that obtained with 2.5 and 5.0 mμgm. of biotin, and that 25 mμgm. of oxybiotin give growth intermediate between that obtained with 5 and 10 mμgm. of biotin. In terms of optical density the activity of oxybiotin is only slightly less than 30 per cent of that of biotin in both cases. Similar results have been obtained with an initial pH of 8.0 in the presence of 0.01 M bicarbonate and a gas mixture of 95 per cent nitrogen and 5 per cent carbon dioxide in a vacuum desiccator. In this experiment the turbidity of the tubes containing oxybiotin was as great as 66 per cent transmission at 24 hours, although the activity compared to biotin at 66 hours was not greater than 25 per cent.

Table III

Relative Activity of Biotin and Oxybiotin in Absence of Aspartic Acid, Initial pH 7.0

Biotin				Oxybiotin			
	Turbidity*				Turbidity*		
	24 hrs.	38 hrs.	66 hrs.		24 hrs.	38 hrs.	66 hrs.
mμgm. per 10 ml.				*mμgm. per 10 ml.*			
0.0	100	99	99	0.0	100	99	99
2.5	86	74	64	2.5	98	94	93
5.0	63	47	42	10.0	96	57	53
7.5	47	36	32	25.0	97	58	36
10.0	39	29	25	250.0	97	52	28
15.0	30	24	20	1,000.0	98	54	27
20.0	26	22	18	5,000.0	97	56	26
25.0	33	22	18	25,000.0	97	97	24

* See foot-note to Table I.

DISCUSSION

The results presented here indicate that oxybiotin is less effective than biotin in causing aspartic acid synthesis. The authors have shown previously (18) that, even in the presence of bicarbonate and biotin, aspartic acid cannot be synthesized at pH 5.8 or below. The data in Tables II and III indicate that the quantitative biotin requirement is largely a function of the pH. Why slightly acid conditions affect oxybiotin activity so much more adversely than that of biotin is not known, although it is presumably related to the effect on bicarbonate concentration. Oxybiotin seems to be inherently less effective than biotin for carbon dioxide fixation even at neutral pH. Under acid conditions, which increase the lag period with biotin, the difference in activity of these two compounds is exaggerated to such an extent that good growth is not attained with oxybiotin during the normal assay period.

Wyss, Lilly, and Leonian (24) have shown that pH has a pronounced effect on the requirement of a *Neurospora crassa* mutant for *p*-aminobenzoic acid. From the dissociation constant of the vitamin and their experimental data, the authors conclude that only the molecular form can be utilized by this organism. This does not appear to explain the effect of pH on oxybiotin activity.

SUMMARY

With an initial pH of 6.5 oxybiotin is rather ineffective in causing aspartic acid synthesis and the growth of *Lactobacillus arabinosus* 17-5 is not more than one-fifth of the maximum regardless of the level of oxybiotin. When the initial pH is 7.0 or more, excellent growth results and oxybiotin is about 30 per cent as active as biotin.

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TRIPHOSPHOPYRIDINE NUCLEOTIDE-CYTOCHROME *c* REDUCTASE IN LIVER

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In yeast the reduction of ferricytochrome *c* by reduced triphosphopyridine nucleotide ($TPNH_2$) is catalyzed by a flavoprotein, cytochrome *c* reductase, which contains flavin mononucleotide (FMN, riboflavin phosphate) as the prosthetic group (1). In animal tissue the reduction of ferricytochrome *c* by $TPNH_2$ has not yet been reported, although the reaction with reduced diphosphopyridine nucleotide ($DPNH_2$) has been observed with pig heart suspensions (2) and liver extracts (3). TPN-cytochrome *c* reductase has now been isolated from pig liver[1] and identified as a flavoprotein with flavin-adenine dinucleotide (FAD) as the prosthetic group. The specific activity of the purified enzyme is the same as that of yeast cytochrome *c* reductase. In the present report the details of the isolation procedure and the properties of the purified liver enzyme are described.

Test Methods and Materials

Test System—Enzyme activity was determined essentially as previously described (1) in a test system consisting of glucose-6-phosphate, *Zwischenferment*, TPN, and cytochrome *c*, as shown in Table I. The following reactions are involved.

$$\text{Glucose-6-phosphate} + \text{TPN} \xrightarrow{\textit{Zwischenferment}} \text{6-phosphogluconic acid} + TPNH_2$$

$$TPNH_2 + 2 \text{ ferricytochrome } c \xrightarrow{\text{cytochrome } c \text{ reductase}} \text{TPN} + 2 \text{ ferrocytochrome } c$$

Before addition of the cytochrome *c* reductase and cytochrome *c*, the reaction mixture was incubated for 15 minutes at room temperature to permit complete reduction of the TPN. Cytochrome *c* reductase activity in the *Zwischenferment* preparation prevented the use of larger quantities to accelerate the reduction. Corrections for this activity were made from blank determinations without added cytochrome *c* reductase. The rate of formation of ferrocytochrome *c* was followed at 549.5 mμ with the Beckman model DU spectrophotometer, with a nominal band width of 0.5 mμ. Although the reduction appeared to follow a first order course, it was nearly linear in the early stages, as shown in Table I. The unit of enzyme activity was defined as the quantity which produced a density change of 1.0 per minute, calculated from the density change observed in the first 2 minutes. The specific activity is the number of units per mg. of protein.

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[1] A preliminary report of this work was presented at the Fortieth annual meeting of the American Society of Biological Chemists at Detroit, 1949 (4).

Protein was determined by the turbidimetric method of Bücher (5). The turbidity, measured at 340 mμ with the Beckman spectrophotometer, was standardized on the basis of the dry weight and nitrogen content of a purified enzyme preparation.

TABLE I

Test for Cytochrome c Reductase Activity

The test system contained 0.10 cc. of glucose-6-phosphate, 25 μM per cc.; 0.02 cc. of *Zwischenferment*, 4 mg. per cc.; 0.05 cc. of TPN, 1.1 μM per cc.; 0.06 cc. of cytochrome *c*, 4×10^{-4} M; 0.04 cc. of cytochrome *c* reductase, 0.29 γ, and 1.25 cc. of 0.05 M phosphate buffer, pH 7.55. Temperature, 23–26°; length of cell, 1.0 cm.; $\lambda = 549.5$ mμ; Beckman spectrophotometer.

Time	Density	ΔFe^{+++}*
sec.		$\mu M \times cc.^{-1} \times min.^{-1}$
0		(0.0033)†
30	0.218	
		0.00306
60	0.248	
		0.00256
90	0.273	
		0.00224
120	0.295	
		0.00204
150	0.315	

* The change in concentration of ferricytochrome *c* (Δ Fe^{+++}) was calculated from the equation $(d_{n+1} - d_n)/(1.96 \times 10^4)$, where d_{n+1} and d_n are successive density readings and 1.96×10^4 represents the difference in the extinction coefficients for ferro- and ferricytochrome *c*.

† Extrapolated to zero time.

Glucose-6-phosphate—This was synthesized by the method of Fischer and Lardy (6). The final product, obtained as the Ba salt, contained 7.40 per cent P and no inorganic P; the purity based on the P content was 94 per cent. The purity was also determined by the reduction of TPN. 39.3 γ of the Ba salt of glucose-6-phosphate, containing 9.4×10^{-3} μM (micromole) of P, brought about the reduction of 8.7×10^{-3} μM of TPN. Of the total P, 93 per cent is therefore present as glucose-6-phosphate. For the enzyme assays barium was removed by addition of the calculated amount of K_2SO_4.

Zwischenferment—Crude *Zwischenferment* was prepared from Anheuser-

Busch brewers' yeast dried at low temperature, according to the procedure of Warburg and Christian (7). In order to obtain a product with low cytochrome *c* reductase content, it was necessary to study the relative activities of the two enzymes in autolysates prepared under various conditions. High temperatures appeared to favor the destruction of the cytochrome *c* reductase; autolysis for 6 hours at 40° was optimal for the lot of yeast used. The dry preparation obtained was about 2 per cent as active as the purified preparation described by Negelein and Haas (8).

Triphosphopyridine Nucleotide—TPN of purity 0.55 was prepared from liver by a modification of the method of Warburg, Christian, and Griese (9). The purity was determined spectrophotometrically with 6.24×10^6 sq. cm. $\times$ mole^{-1} as the extinction coefficient of $TPNH_2$ at 340 mμ (10).

Cytochrome c—This was prepared from beef heart by the procedure of Keilin and Hartree (11).

Other Substances—FAD was prepared from yeast by the method of Warburg and Christian (12). FMN was obtained from the dinucleotide by hydrolysis with nucleotide pyrophosphatase from potato (13). 0.2 mg. of FAD in 2.0 cc. of H_2O was treated with 0.01 cc. of nucleotide pyrophosphatase containing 0.22 mg. of protein per cc. (280 units per cc.). The mixture was incubated at 34° for 30 minutes, when the fluorescence had increased to a maximal value. The solution thus obtained (FMN) was stored at 0°. The protein of D-amino acid oxidase was prepared according to the procedure of Negelein and Brömel (14) through Step E. DPN was prepared by the method of Williamson and Green (15) and reduced by the method of Ohlmeyer (16). Lactic dehydrogenase was a preparation from rabbit muscle (17).

Purification of Enzyme

Preparation of Acetone Powder—Liver was obtained from freshly slaughtered pigs, sliced into thin strips, and chilled in crushed ice. 100 gm. portions were homogenized in Waring blendors with 500 cc. of acetone cooled to −10°. The residue was filtered with suction, again homogenized with cold acetone, filtered, and dried at room temperature. The powder was stored at 2° and used within 2 weeks. It was important to select liver which was pale in appearance, since dark red liver contributed substances which interfered with the ammonium sulfate and pH fractionations, greatly reducing both yield and purification.

Extraction—50 gm. of acetone powder were suspended in 750 cc. of 0.1 M Na_2HPO_4 and shaken gently for 10 minutes at 2°. The suspension was centrifuged and the supernatant, which contained most of the soluble proteins but negligible cytochrome *c* reductase activity, was discarded. The precipitate was vigorously homogenized in a Potter-Elvehjem homogenizer (18) with small additions of 0.1 M Na_2HPO_4 until about 50 cc. had been added and a smooth brown paste obtained. The thick homogenate was added to 700 cc. of 0.1 M Na_2HPO_4, mixed, and centrifuged for 5 minutes at 2500 R.P.M. The supernatant was poured off through fine gauze. About 700 cc. of a light brown suspension were obtained which could be kept for several days without loss of activity (acetone powder extract) (Table II).

Digestion with Trypsin, Ammonium Sulfate Fractionation—4.08 liters of extract from 300 gm. of acetone powder were treated with 1.32 gm. of trypsin (Wilson, 1:300) and incubated for 30 minutes at 34°. The mixture was cooled to 2° and treated with 258 gm. of ammonium sulfate per liter. After 30 minutes, the precipitate was centrifuged and discarded. The supernatant was treated with 342 gm. of ammonium sulfate per liter. The precipitate was collected on a Büchner funnel, dissolved in 600 cc. of water, and dialyzed for 18 hours against 0.04 M sodium acetate. The pH of the acetate solution was 6.8 to 7.2, owing to residual acid in the demineralized water used in its preparation. The dialyzed solution lost about 10 per cent of its activity when stored overnight at 2° (Ammonium Sulfate I, Table II).

TABLE II

Preparation of Cytochrome c Reductase

Step	Total units*	Specific activity
		units per mg.
I. Acetone powder extract (from 300 gm. powder)	10,200	0.11
II. Ammonium Sulfate I	8,660	1.71
III. pH ppt	5,910	6.4
IV. Calcium phosphate gel eluate	5,280	24
V. Ammonium Sulfate II	3,690	70
VI. Aluminum hydroxide gel eluate	2,630	140
VII. Repeat Steps V and VI		140–160

* Density change per minute.

pH Precipitation—Since the pH range for optimal precipitation of the enzyme in the dialyzed solution varied considerably from preparation to preparation, it was necessary to make a small pilot run in each case. With 30 cc. (Ammonium Sulfate I), fractions were collected at pH 5.3, 5.1, 4.9, and 4.7. For the preparation described here most of the enzyme was precipitated between pH 5.3 and 4.9. The dialyzed solution (540 cc.) was brought to pH 5.26 with 39 cc. of 0.2 N acetic acid and after 5 minutes at 2° the solution was centrifuged and the precipitate discarded. The supernatant solution was acidified to pH 4.87 with 53 cc. of 0.2 N acetic acid, again kept 5 minutes, and then centrifuged. This precipitate was dissolved in water with the aid of 11.4 cc. of 0.2 N NH_4OH; the volume was 153 cc. and the pH about 8.0. To prepare the enzyme for the calcium phosphate gel step, it was precipitated with 95 gm. of ammonium sulfate. The precipitate was dissolved in water and adjusted to about pH 8.0 with 4.0 cc. of 2 N NH_4OH (pH precipitate, 150 cc.).

Calcium Phosphate Gel Adsorption and Elution—Calcium phosphate gel (19), aged 3 to 6 months, was added until the supernatant was pale pink, with all the yellow components adsorbed. 150 cc. of the pH precipitate required 220 cc. of the gel (dry weight 8.8 mg. per cc.). The gel was centrifuged and was washed two times in the centrifuge with 60 cc. of 0.01 M phosphate buffer, pH 7.4, and the enzyme was eluted four times with 60 cc. of 0.1 M K_2HPO_4 (calcium phosphate gel eluate).

Lyophilization—240 cc. of eluate were precipitated with 167 gm. of ammonium sulfate. The precipitate was dissolved in 30 cc. of 0.15 M pyrophosphate buffer, pH 7.6, and lyophilized. 3.0 gm. of powder were obtained containing 150 mg. of protein. The powder was stable for several months at 3° *in vacuo*.

Alkaline Ammonium Sulfate Fractionation—Since the concentration of ammonium sulfate at which optimal precipitation occurred was somewhat variable, a number of fractions were collected, tested separately, and the best ones pooled for the subsequent steps. 3.0 gm. of powder were dissolved in 200 cc. of H_2O and treated with 76 gm. of ammonium sulfate and 4.0 cc. of concentrated ammonia water to bring the pH to about 8.0. The precipitate was collected by centrifugation and dissolved in 15 cc. of water. The supernatant was treated with 7.7 gm. of ammonium sulfate, the precipitate was collected and dissolved, and the supernatant again treated with 7.7 gm. of ammonium sulfate. In this way a total of six fractions was collected of which the third, fourth, and fifth were combined (Ammonium Sulfate II, 48 cc.).

Aluminum Hydroxide Gel Adsorption and Elution—The combined ammonium sulfate fractions were diluted with 40 cc. of water and treated with 40 cc. of aluminum hydroxide gel $C\gamma$ (20) aged 6 months or longer (dry weight 11.4 mg. per cc.). The gel was centrifuged and washed twice with 10 cc. portions of 0.025 M phosphate buffer, pH 7.2, and the enzyme was eluted six times with 10 cc. portions of 0.1 M phosphate buffer, pH 7.2 (aluminum hydroxide gel eluate).

Further Purification—A further small purification was usually accomplished by repetition of the last two operations, alkaline ammonium sulfate and aluminum hydroxide gel adsorption; the final specific activity was usually 140 to 160 units per mg. The purified enzyme was unstable in solution and slowly lost activity when frozen and stored at −20°, or when lyophilized as described above. In 20 minutes at 34° one-third of the activity was destroyed.

Absorption Spectrum

Solutions of purified cytochrome *c* reductase with specific activity of 150 to 160 units per mg. are bright yellow and show the characteristic flavoprotein absorption spectrum (Fig. 1), with maxima at 275, 380, and 455 mμ. With less pure preparations a strong hemin band at about 410 mμ completely obscures the flavoprotein spectrum; this component was removed in the last purification step. On reduction with excess $TPNH_2$ the absorption at 455 mμ was largely but not entirely removed. The purity of the enzyme preparation may be estimated from the content of reducible flavoprotein. The change in density observed after addition

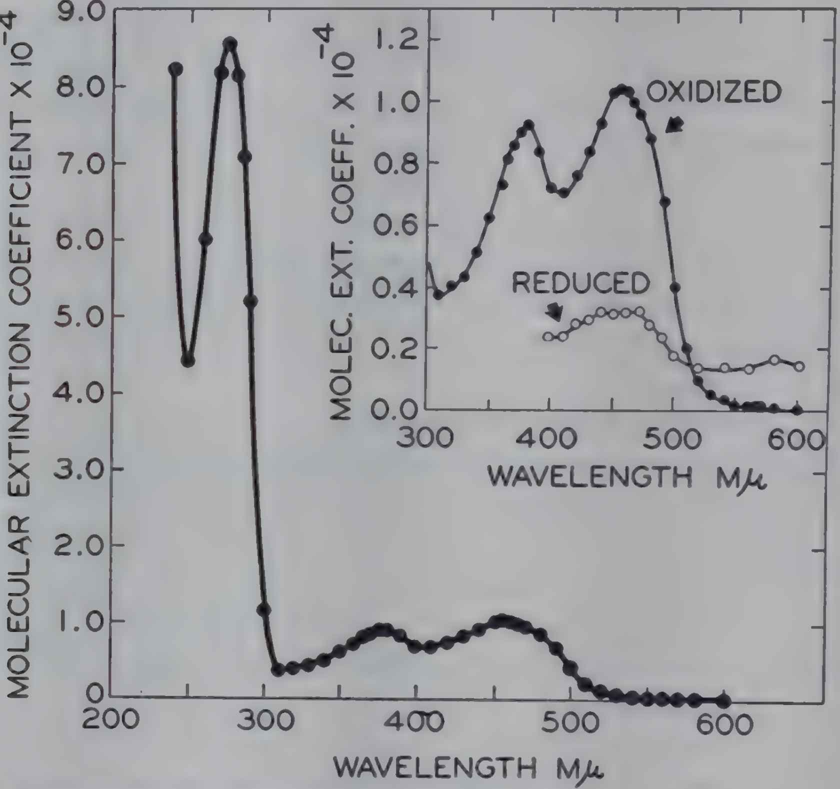

FIG. 1. The absorption spectrum of cytochrome *c* reductase. Specific activity = 162 units per mg. The extinction coefficients were based on a value of 1.04×10^6 sq. cm. × $mole^{-1}$ at 455 mμ. The reduced spectrum was obtained by the addition of 0.20 mg. of *Zwischenferment*, 1.25 μM of glucose-6-phosphate, and 0.03 μM of TPN in a total volume of 0.3 cc. In the insert the portion between 300 and 400 mμ is enlarged to show the band details.

of excess TPN, *Zwischenferment*, and glucose-6-phosphate in the experiment described in Fig. 1 was 0.071. With the assumption that the extinction coefficient for flavoproteins at 455 mμ is 1.04×10^6 sq. cm. $\times$ mole^{-1} (1, 21, 22), the concentration of cytochrome *c* reductase was 6.8×10^{-8} mole per cc. The total protein present was 4.6×10^{-4} gm. per cc., from which the molecular weight was calculated to be 68,000. This result is in agreement with molecular weights of 72,000, 65,000, and 78,000 obtained from the flavin content for "old" yellow enzyme (21), "new" yellow enzyme (22), and yeast cytochrome *c* reductase (1), respectively, and of 83,000 and 78,000 for "old" yellow enzyme determined by ultracentrifugation (23, 24). Since on this basis cytochrome *c* reductase accounted for practically all of the protein present, the residual absorption after reduction was due to a contaminant of high specific absorption.

Prosthetic Group

Splitting and Resynthesis—Cytochrome *c* reductase was split by treatment with acid in the presence of ammonium sulfate as described by Warburg and Christian (12). 4.0 cc. of aluminum hydroxide gel eluate (1.80 mg. of protein) were treated with 0.9 gm. of ammonium sulfate and 0.4 cc. of 1.0 N H_2SO_4 containing 0.22 gm. of ammonium sulfate per cc. After 10 minutes at 0°, the precipitate was centrifuged and dissolved in 2.0 cc. of 0.1 M phosphate buffer, pH 7.0. The supernatant after neutralization with 0.4 cc. of 0.2 N NH_4OH had a volume of 6.5 cc. These solutions will be referred to as Protein R and Flavin R, respectively.

The reactivation of the split protein was studied with FAD and FMN prepared as described in the first section, with results as shown in Fig. 2.[2]

The residual activity of Protein R was increased by either FAD or FMN and the mononucleotide was considerably more effective than the dinucleotide. Although the data do not permit a precise determination of the dissociation constants, it is apparent that half reactivation was obtained with FMN at about 1×10^{-8} M and with FAD at about 2×10^{-8} M.

Effect of Nucleotide Pyrophosphatase on Cytochrome c Reductase Flavin—While the results of the preceding section do not permit any conclusion as to the identity of the cytochrome *c* reductase prosthetic group, the experiments described below identify the flavin obtained from cytochrome *c* reductase as FAD.

Mixtures of FAD and FMN can be analyzed fluorometrically by the method of Burch, Bessey, and Lowry (25), based on the increase in fluorescence which accompanies the splitting of FAD to FMN and adenylic acid. The results with Flavin R are compared with those obtained with FAD in Table IV. Since the fluorescence of FAD is 0.14 times that of FMN, the per cent of FAD in a mixture is given by the equation

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[2] With a synthetic sample of FMN kindly furnished by Dr. C. W. Sondern of the White Laboratories, Inc., Newark, New Jersey, reactivation of cytochrome *c* reductase protein was about equal to that given with FMN prepared by the hydrolysis of FAD.

$$\% \text{ FAD} = \frac{f_2 - f_1}{0.86 f_2} \times 100 \tag{1}$$

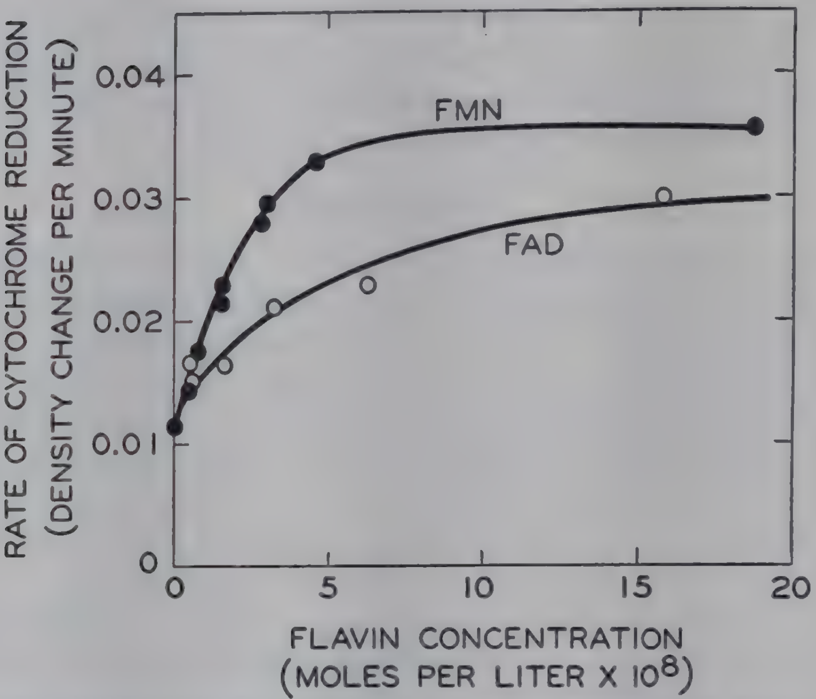

Fig. 2. The reactivation of cytochrome *c* reductase protein by flavins. 0.05 cc. of Protein R was incubated for 10 minutes at 0° with flavin in 0.01 M phosphate buffer in a total volume of 0.8 cc. The activity was tested as in Table I, with 0.04 cc. of incubation mixture. The flavin concentrations are the final concentrations in the test.

where f_1 and f_2 are the fluorometer readings before and after hydrolysis of the flavin mixture. In the experiment in Table III, nucleotide pyrophosphatase was used to hydrolyze the dinucleotide. As shown in Table III, all the flavin in Flavin R is accounted for as FAD.

In Table IV is shown the effect of hydrolysis of FAD and Flavin R on the reactivation of Protein R. The activities before and after hydrolysis were compared with the reactivation obtained with an excess of FMN. As would be expected from Fig. 2, FAD was more effective after hydrolysis. A similar increase in activity was observed with Flavin R. In the last line of Table IV is included an experiment with intact cytochrome *c* reductase.

In agreement with results obtained with D-amino acid oxidase (13), the bound flavin was not attacked by nucleotide pyrophosphatase under conditions which resulted in complete hydrolysis of the free dinucleotide.

Activity of Cytochrome c Reductase Flavin in D-*Amino Acid Oxidase Test*—Further evidence for the identity of the flavin of cytochrome *c* reductase was provided by its activity with the protein of D-amino acid oxidase. The rate of production of pyruvate from D-alanine was measured by the oxidation of $DPNH_2$ in the presence of lactic dehydrogenase. The test system contained 33 µM of DL-alanine, 0.10 µM of $DPNH_2$, 22 γ of lactic dehydrogenase, and 56 γ of amino acid oxidase (Protein E) in 0.03 M pyrophosphate buffer, pH 8.3, in a final volume of 1.6 cc. The oxidation

TABLE III

Fluorometric Analysis of Cytochrome c Reductase Flavin

4.7×10^{-4} µM of flavin was incubated for 30 minutes at 34° with 10 units of nucleotide pyrophosphatase in 1.0 cc. of 0.05 M phosphate buffer, pH 7.4. The control solution (before hydrolysis) was incubated without nucleotide pyrophosphatase. Galvanometer readings.

	Flavin R	FAD
Before hydrolysis (f_1)	5.0	11.0
After hydrolysis (f_2)	32.5	45.5
% FAD*	98	93

* Calculated from Equation 1.

TABLE IV

Flavin Activity with Cytochrome c Reductase Protein

The flavins were incubated for 30 minutes at 34°, with 10 units of potato nucleotide pyrophosphatase in 0.75 cc. of 0.01 M phosphate buffer, pH 7.2. After incubation the solutions were cooled, 0.05 cc. of Protein R was added, and the incubation was continued for 15 minutes at 0°. Controls (before splitting) were incubated without nucleotide pyrophosphatase. Tests were made with 0.04 cc. of the incubation mixture as in Table I.

Flavin	Before hydrolysis		After hydrolysis	
	Specific activity	Per cent reactivation	Specific activity	Per cent reactivation
	units per mg.		*units per mg.*	
None	0.009	0		
FMN (5.7 µM $\times 10^{-3}$)	0.031	100		
FAD (3.8 µM $\times 10^{-4}$)	0.016	32	0.029	91
Flavin R (3.7 µM $\times 10^{-4}$)	0.020	50	0.032	105
Cytochrome *c* reductase	0.020		0.022	

of $DPNH_2$ was measured with the Beckman spectrophotometer at 340 mμ. The rate of oxidation of $DPNH_2$ was not linear, but increased as the oxidation progressed. Reproducible results were obtained, however, by measuring the time required for the density to change between two defined values, in this case 0.325 and 0.225. The reciprocal of the time is a measure of the FAD content. The concentration of FAD required for half activation of D-amino acid oxidase protein by this method agreed with that obtained by the manometric technique.

Solutions of cytochrome *c* reductase were analyzed for their FAD content by this procedure after the flavin was liberated by heating at 100° for 5 minutes. The results are shown in Table V. In the case of Preparation II, the one used in the determination of the molecular weight (Fig. 1), there is excellent agreement between FAD and flavoprotein content, the values in both cases being about 70 per cent of the total light absorption at 455 mμ. With two other preparations of cytochrome *c* reductase, the FAD content accounted for 65 and 80 per cent of the total absorption at 455 mμ. These results are consistent with the conclusion that the residual absorption after reduction with $TPNH_2$ is due to a contaminant of high specific absorption and that FAD is the prosthetic group of liver cytochrome *c* reductase.

TABLE V

FAD Content of Cytochrome c Reductase

Preparation No.	Total flavin calculated from absorption at 455 mμ	Flavin calculated from reduction with $TPNH_2$	FAD*
	μM per cc.	*μM per cc.*	*μM per cc.*
I	0.0082		0.0053
II	0.0088	0.0068	0.0063
III	0.0035		0.0028

* Determined in amino acid oxidase test.

Activity of Liver Cytochrome c Reductase

Turnover Number—From the data in Table I the turnover number for liver cytochrome *c* reductase is 1140 moles of cytochrome *c* per mole of flavoprotein per minute, with the molecular weight taken to be 68,000. For the yeast enzyme under almost identical test conditions, the turnover number was calculated to be 1300 (molecular weight = 78,000). Thus, despite different prosthetic groups, the catalytic activities of the yeast and liver enzymes are nearly the same.

The reaction of liver cytochrome *c* reductase with oxygen is less than 2 per cent as fast as the reaction with cytochrome *c*.

Under the conditions of the test the rate of cytochrome *c* reduction was independent of both TPN and cytochrome *c* concentration. Although the reduction appears to follow a first order course with respect to ferricytochrome *c*, this was not confirmed by variation of the total cytochrome *c* concentration, since an increase of 6-fold resulted in the same initial rate of reduction. The reaction was thus zero order with respect to cytochrome *c* and the decline in rate with time must be attributed to some other factor. The substrate optima for both TPN and cytochrome *c* are at concentrations below 10^{-5} M.

Association of Cytochrome c Reductase with Liver Particles—Cytochrome *c* reductase activity in the acetone powder extract is associated with insoluble particles. In this form the activity is quantitatively precipitated by ammonium sulfate below 40 per cent saturation. As a result of tryptic digestion the milky suspension becomes darker and more opalescent, the protein content falls by one-third to one-half, and 90 per cent saturation with ammonium sulfate is required for complete precipitation.

Homogenates of fresh liver, when fractionated according to the procedure of Hogeboom, Schneider, and Pallade (26), have cytochrome *c* reductase activity in the mitochondrial fraction. The specific activity of these particles is the same as that of the acetone powder extract.

The content of cytochrome *c* reductase in liver is comparable with that observed in ale yeast. 300 gm. of acetone powder derived from about 3 kilos of fresh liver yielded about 65 mg. of enzyme. From 300 gm. of dried ale yeast about 180 mg. of enzyme were obtained.

The activity of DPN-cytochrome *c* reductase in crude pig liver extracts is about 20 times that of the TPN enzyme. This activity is almost completely absent after the first ammonium sulfate fractionation.[3]

Optimal pH—The optimal pH for liver cytochrome *c* reductase is shown in Fig. 3. There is a marked fall in activity above pH 8.4 and below pH 7.5, with negligible activity remaining at pH 6.0. From the data in Fig. 3 it is evident that there is no requirement for added phosphate in the reaction, since rates obtained in the absence of phosphate agree with those obtained in phosphate buffer. 2,4-Dinitrophenol at a concentration of 1.3×10^{-4} M did not affect the activity.

DISCUSSION

Ever since the isolation of the "old" yellow enzyme by Warburg and Christian (7), flavin enzymes have been assigned an important rôle in the oxidation of the pyridine nucleotides. There was some doubt, however, as to the requirement for accessory enzymes which would link them to cytochrome *c*, since none of the early flavoproteins, such as the "old" yellow enzyme, "new" yellow enzyme (22), or heart flavoprotein (27), react with cytochrome *c* or with oxygen at rates consistent with physiological requirements for respiration. With the isolation of cytochrome *c* reductase from yeast (1), it was demonstrated that a flavoprotein could react directly with cytochrome *c* at a rapid and physiological rate. No other intermediate was found nor was it necessary to postulate one. In the present paper a similar enzyme from animal tissue is described. While the liver enzyme differs from that isolated from yeast in that FAD appears to be the prosthetic group, it is of interest that with FMN a synthetic enzyme is obtained having a higher activity. With both "old" yellow enzyme (28) and yeast cytochrome *c* reductase[4] synthetic enzymes could be prepared in which the FMN is replaced by FAD. In both cases the "FAD enzyme" is only one-third to two-thirds as active as the "FMN enzyme."

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[3] Unpublished observations by L. A. Heppel.

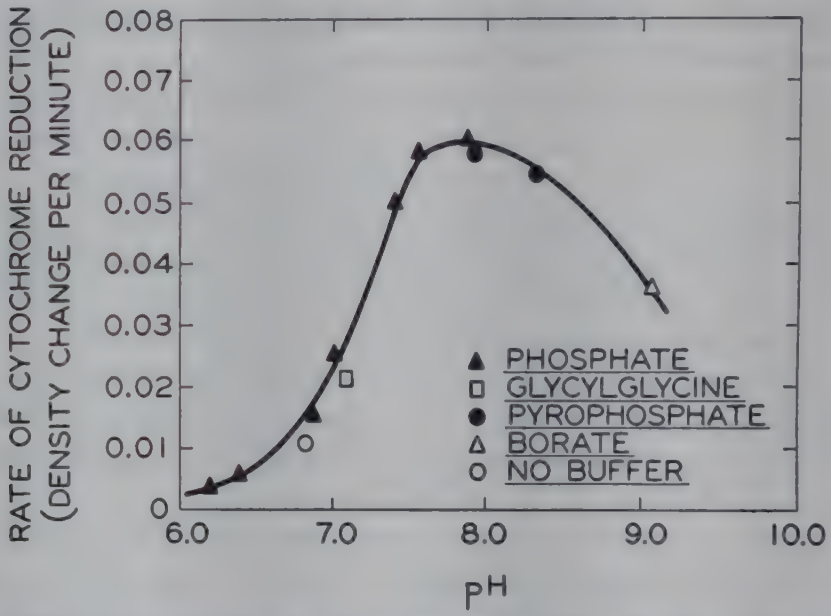

FIG. 3. Activity of cytochrome *c* reductase as a function of pH. The buffers were all 0.04 M. Test as in Table I with 0.7 mg. of cytochrome *c* reductase; specific activity = 54.

The author is indebted to Dr. L. A. Heppel for $DPNH_2$ and amino acid oxidase protein, to Dr. A. Kornberg for preparations of nucleotide pyrophosphatase and lactic dehydrogenase, and to Dr. A. Schrecker for FAD. Thanks are also due to Miss Pauline Smyrniotis for valuable technical assistance.

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[4] Unpublished observations.

SUMMARY

1. TPN-cytochrome *c* reductase has been isolated in essentially pure form from pig liver acetone powder.

2. The spectrum is that of a flavoprotein, with maxima at 275, 380, and 455 mμ. The molecular weight calculated from the flavin content is 68,000.

3. The enzyme can be split into protein and flavin fractions. The protein is reactivated by either flavin mononucleotide (FMN) or flavin-adenine dinucleotide (FAD).

4. From its hydrolysis with nucleotide pyrophosphatase and activity in the D-amino acid oxidase system, the flavin of cytochrome *c* reductase appears to be FAD.

5. The specific activity and concentration of liver cytochrome reductase are comparable with those of cytochrome *c* reductase in yeast.

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THE LINKAGE OF THE AMINO GROUP IN HEPARIN*

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Alleged Acetyl Content of Heparin

Many erroneous assumptions have been made from time to time as to the chemical nature and the composition of heparin. To these we must now add the assumption (1–6) that heparin contains an acetyl group, which at one time seemed fairly well justified. As pointed out by Fürth and coworkers (7), the amino sugar is monoacetylated in all the known mucopolysaccharides and in chitin. Thus, acetic acid occurs in chondroitinsulfuric acid (8), in the hyaluronic acid of Meyer (3), in the immunopolysaccharide of type I pneumococci (9), and in the blood group substances (10, 11). The first analytical findings on heparin also supported this view.

The analytical methods applied, however, soon proved somewhat unreliable. The Kuhn-Roth (12) alkaline hydrolysis in methyl alcohol, applied in the first communication (1), later proved, when applied to chondroitinsulfuric acid (2), to liberate a large excess of acid, as found simultaneously by Friedrich and Sternberg (13). When, on the contrary, the toluenesulfonic acid was used for hydrolysis, either as recommended by Friedrich and Rapoport (14) or as modified by Friedrich and Sternberg, no acetic acid whatsoever was found in heparin.

Taking into account the extreme slowness with which reducing groups are liberated on acid hydrolysis of heparin, Jorpes and Bergström, who unfortunately did not follow the liberation of the amino group quantitatively, thought that strong hydrolytic agents were necessary, such as 10 per cent (by volume) of sulfuric acid. In fact, acetic acid was also identified by them as silver salt in the distillate after acid hydrolysis.

In the meantime a negative finding was made by Masamune, Suzuki, and Kondoh (15) and by Wolfrom and coworkers (16). The latter authors applied both the toluenesulfonic acid method and the chromic acid oxidation method for the determination of the CH_3C group.

The findings of the Japanese workers and of Wolfrom and coworkers necessitated a reinvestigation of the question with more reliable methods.

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* This investigation was aided by grants from the Knut and Alice Wallenberg Foundation, Stockholm.

Nature and Amount of Acid Distilled after Acid Hydrolysis of Heparin—At first, the nature of the volatile fatty acid formed on submitting the heparin and related substances to hydrolysis with 10 per cent (by volume) of sulfuric acid during 4 to 48 hours was determined qualitatively by means of the distillation constant, as described by Clark ((17) p. 85). The values for the distillation constant found were formic acid 20.8 ± 0.22, acetic acid 35.6 ± 0.56, and propionic acid 55.8 ± 0.50.

Only acetic acid was liberated from chondroitinsulfuric acid and from a water-soluble fraction of the barium salt of crude heparin. From the less soluble barium salt of heparin a mixture of acetic acid and formic acid was obtained. Starch, glucose, galactose, and glucosamine yielded only formic acid.

TABLE I

Acetic Acid Found When Heparin and Related Substances Were Submitted to Acid Hydrolysis

	Hydrolysis		Acid found			
			Before treatment with HgO		After treatment with HgO	
	Per cent (by volume) of sulfuric acid	Time	Per cent*	Distillation constant	Per cent†	Distillation constant
		hrs.				
Heparin (Na salt), 12.97% sulfur	10	24	3.87	29.3	0.39	36.9
	25	48	13.85		0.47	36.9
	25	24			0.52	36.7
	25	48			0.47	37.9
Chondroitinsulfuric acid (Ca salt)	10	24	7.3	38.8	5.5 (Theoretical 12.1)	34.0
	25	36			4.8	36.2
Chitin	25	48			21.5 (Theoretical 26.8)	38.0
	25	20			7.8	36.5
	10	48			4.1	36.4

* Calculated as acetic acid.

† No correction is made for the loss of acetic acid during the treatment with HgO. In treating mixtures of acetic and formic acids in the same way, the loss varied between 20 and 30 per cent of the acetic acid present.

The next step was to remove the formic acid through oxidation with mercuric oxide (see Clark ((17) p. 89)) and to determine the remaining acetic acid quantitatively by titration. The results are given in Table I.

When submitted to hydrolysis with sulfuric acid, chondroitinsulfuric acid and chitin thus gave considerable amounts of acetic acid. The heparin sample gave about a tenth of the theoretical figure. The small amount of acetic acid found could derive from the polysaccharide related to heparin, which follows the heparin and can be separated from it as a more soluble barium salt.

Content of Acetic Acid As Determined According to Lemieux and Purves—Since the chromic acid method of Kuhn and l'Orsa for the determination of terminal methyl groups in sugars, as modified by Lemieux and Purves (18) for the determination of acetic acid, gave the best results on analyzing chondroitinsulfuric acid, we applied this method to our samples. The results are given in Table II.

The chromic acid method of Lemieux and Purves showed, as did the hydrolysis with acids, a decreasing content of acetic acid with increasing purity of the heparin samples. Almost correct values were found for chondroitinsulfuric acid. In an easily soluble barium salt of the heparin polysaccharide with 7.25 per cent sulfur, 3.05 and 2.59 per cent of acetic acid were found. The low content of acetic acid, less than 1 per cent, found on hydrolyzing with acid a sodium salt of the purer heparin containing 12.97 per cent of sulfur, and about 0.9 per cent found with the chromic acid method on analyzing a barium salt with 10.31 per cent of sulfur, certainly derives from an admixture of impurities. The more soluble barium salts, obtained by fractionating the crude heparin polysaccharide, contain acetic acid, as shown by Jorpes and Gardell (19).

TABLE II

Quantitative Determination of Acetic Acid According to Lemieux and Purves

	Sulfur	Nitrogen	Acetic acid	
	Per cent dry substance	Per cent dry substance	Per cent dry substance	
			Found	Theoretical
Chondroitinsulfuric acid (Ca salt)		1.99	11.5 11.9 11.4	12.1
Heparin (easily soluble barium salt)	7.25	1.86	3.05 2.59	
" (insoluble barium salt)	10.31	2.05	0.92 0.86 0.86	

Determination of Acetic Acid by Means of Transesterification—We also tried the transesterification method of Stuart (20), using an apparatus of the same construction as that used by him, following his description of the method exactly. The formation of volatile ethyl acetate makes this method highly specific and very useful for our purpose. We also followed the liberation of the amino group. The results are given in Table III.

With this method also, 7 per cent of the theoretical figure was obtained for the acetic acid content of chondroitinsulfuric acid after 4 hours distillation. During distillation there was a fairly good correlation between the NH_2 nitrogen and the acetyl liberated. On the other hand, about only 10 per cent of the theoretical figure for the acetyl content of heparin was obtained after 5 hours distillation, although the amino group was totally free after 2 hours.

Linkage of Amino Group

Heparin thus constitutes an exception to the rule that the amino sugars of the animal polysaccharides are monoacetylated. The linkage of the amino group must be explained in another way. There are no NCH_3 groups in heparin (16). Masamune and coworkers suggested an anhydride linkage, $—C—NHSO_2—OC—$ to one of the sulfuric acid groups. This possibility is excluded because all the ester sulfuric acid of heparin is ionized and can be neutralized with alkali. As shown by Wilander (21), the free acid of heparin, prepared through electrodialysis, consumes 4 equivalents of alkali, 3 within the range below pH 3 corresponding to the three sulfuric acid groups and the 4th between pH 3 and 7 corresponding to the carboxyl group. The amino group was not free, as could be shown by the lack of buffering capacity between pH 7 and 10. The same was found by Wolfrom and coworkers (16). The Ba:S ratio of their acid barium salt was 1:2 and the carboxyl group could be titrated quantitatively.

TABLE III

Liberation of NH_2 Nitrogen and Acetic Acid from Chondroitinsulfuric Acid and Heparin in Transesterification Process, As Modified by Stuart

	Time	NH_2 nitrogen	Acetic acid	
			Found	Theoretical
	hrs.	*per cent*	*per cent*	*per cent*
Chondroitinsulfuric acid (Ba salt)	2	1.44	4.08	12.1
	3	1.70	5.78	
	4	2.03	8.40	
Heparin, Ba salt (9.25% sulfur)	2	1.76	0.28	
	5	1.76	0.69	

Wolfrom and coworkers (16) discussed the different possibilities which could be considered and found a linkage to the sugar aldehyde group possible. Such a linkage is, however, highly improbable because of the extremely slow liberation of reducing groups during acid hydrolysis of heparin. When boiled with 7.5 per cent (by volume) of sulfuric acid for 5 to 10 minutes, heparin liberates reducing groups, calculated as glucose, corresponding to only 1 to 2 per cent of the organic material (2). In fact this principle has been used later (19) to differentiate heparin and the polysaccharide related to it from hyaluronic acid and chondroitinsulfuric acid, which are both easily hydrolyzed. Wolfrom and McNeely (22) made the same observation regarding the absence of reducing groups. In our experiments the amino group of heparin was liberated through boiling with 0.04 N hydrochloric acid for about 3 hours without any reducing capacity being found. Nor were any volatile acids or aldehydes liberated. Consequently, the aldehyde group of the uronic acid moiety of heparin cannot be linked to the amino group.

Sulfuric Acid Bound to Amino Group

Wolfrom and McNeely (22) paid due attention to the possibility of a sulfate group being bound to the amino group. They could not, however, find any correspondence in the amounts of sulfuric acid and amino nitrogen liberated during acid hydrolysis, the former being only 50 per cent the expected value. As is seen in Table IV, we also found no strict relationship, the amount of sulfuric acid found in the beginning being too low. After half an hour it tended to exceed the amount of amino nitrogen.

TABLE IV

Relative Amounts of NH_2 Nitrogen and Sulfuric Acid Split Off from Heparin at 100° in 0.04 N HCl

Time	NH_2 nitrogen	$—SO_2OH$
min.	*m.eq.*	*m.eq.*
7	16.4	Trace
15	27.8	14.1
45	68.5	76.5
60	74.5	94.5

The first opalescence of barium sulfate appeared when about 12 per cent of the nitrogen in heparin was set free as NH_2. At this stage, and even later, it was not possible to separate the barium sulfate quantitatively. The heparin polysaccharide kept it in a colloidal state. It could first be separated when the heparin molecule had been depolymerized. We made use of the greater stability of the nitrogen linkage in alkaline solution to reach this goal.

Whereas the nitrogen of heparin is set free as NH_2 nitrogen in 0.04 N HCl at 100° for about 3 hours, no NH_2 nitrogen, or very little, is set free on boiling in normal sodium hydroxide solution for 1 hour. Ester sulfates are liberated corresponding to about 25 per cent the total. 75 per cent of the anticoagulant activity is destroyed in 5 minutes and practically all in 1 hour. The appearance of the amino group in alkaline solution therefore bears no relationship to the disappearance of the anticoagulant activity.

The sodium salt obtained after boiling heparin in N NaOH for 1 hour contained 9.5 per cent sulfur, 2.9 per cent total nitrogen (calculated about 2 per cent), and 0.31 per cent NH_2 nitrogen. At least 85 per cent the nitrogen of heparin was still bound in the original linkage. The starting material was a sodium salt with 12.2 per cent sulfur, 2.14 per cent nitrogen, and 0.24 per cent amino nitrogen. After alkaline hydrolysis the solution was cooled and neutralized with HCl, dialyzed against distilled water, concentrated, and treated with alcohol. When this preparation was submitted to acid hydrolysis in 0.04 N HCl, exactly equivalent amounts of sulfate and NH_2 nitrogen were liberated, as is seen in Fig. 1.

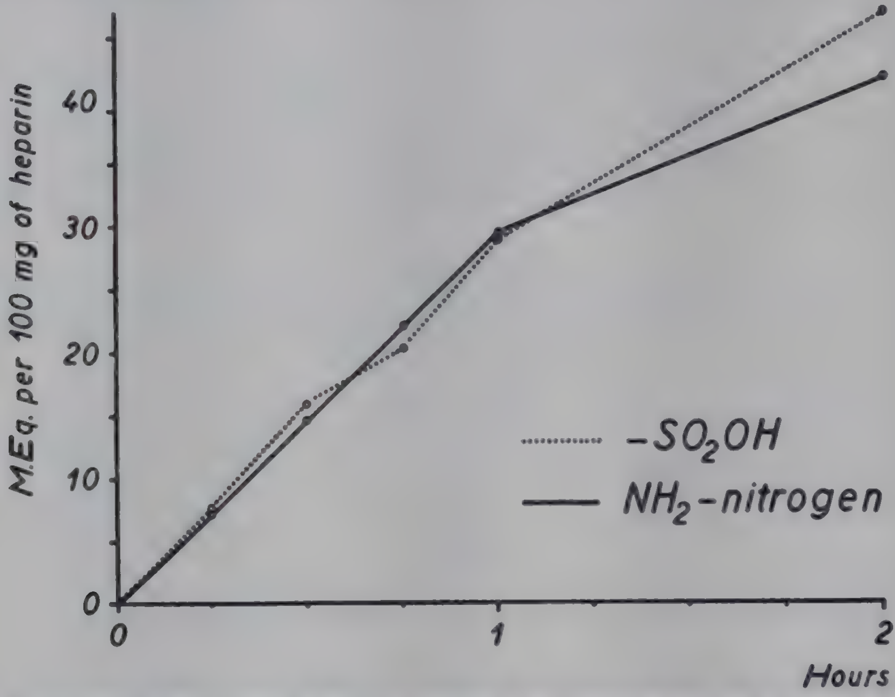

FIG. 1. Relative amounts of amino nitrogen and sulfur split off when a 2 per cent solution of the alkali-treated heparin was boiled in 0.04 N HCl. The amount of nitrogen set free as NH_2 nitrogen during 2 hours corresponds to 30 per cent of the calculated nitrogen content of the alkali-treated heparin.

The acid hydrolysis was repeated four times with three different samples of the depolymerized, partly hydrolyzed heparin.

In the hydrolysis shown in Fig. 1, 21 per cent of the nitrogen calculated to belong to the heparin was set free as NH_2 nitrogen in 1 hour and 30 per cent in 2 hours. In another hydrolysis, 27 per cent of the nitrogen was liberated in 30 minutes and 35 per cent in 1 hour. After 30 minutes the amounts were 39 m.eq. of NH_2 nitrogen and 34 m.eq. of $—SO_2OH$ and, after 1 hour, 49 m.eq. of NH_2 nitrogen and 46 m.eq. of $—SO_2OH$.

In two other hydrolyses a smaller effect was obtained because the solution, 3.5 per cent, was more concentrated, giving a weaker acidity, and the volume was a little larger. Here the first trace of NH_2 nitrogen appeared after 15 minutes simultaneously with traces of free sulfates. After 30, 45, and 60 minutes identical figures were obtained for the two entities, although only 5 per cent of the nitrogen was liberated. On prolonged acid hydrolysis the figures for the sulfur equivalent slightly exceeded those for the NH_2 nitrogen equivalent, at 2 hours by 10 per cent and at 8 hours by 20 per cent, at which time 60 per cent of the nitrogen was set free as amino nitrogen.

The figures for the amino nitrogen are correct within 2 to 3 per cent. The double analysis for sulfur usually checks within the same range, but in this case the variations were greater, *i.e.* 5 to 10 per cent.

DISCUSSION

Acetic acid cannot occur in heparin, as is shown by different methods of analysis, which gave good yields of acetic acid when ordinary mucopolysaccharides were analyzed. Nor can the amino group be involved in any linkage to the aldehyde group of the glucuronic acid or of the glucosamine itself because no aldehyde groups appear simultaneously with the NH_2 nitrogen. A linkage to sulfate groups must therefore be considered.

In fact, free sulfuric acid does under certain conditions appear simultaneously with the amino nitrogen with exactly the same speed and in equimolecular amounts. This could, of course, be only coincidental, but the similarity in the rates of hydrolysis is so close that a linkage between the two groups is highly probable. Consequently, the amino group should occur in a $—NHSO_2OH$ linkage as a substituted amidosulfuric or sulfamic acid. The properties of these compounds also closely resemble those of heparin. The amidosulfuric acid, first isolated by Berglund in Lund, Sweden, 1875 (23–25), is very labile in acid solution and fairly resistant in the presence of alkali.

This is also true of the amino linkage in heparin. It is disrupted at 100° in 0.04 N HCl in 3 hours, whereas it resists heating in N NaOH for 1 hour. When it breaks up in acid solution, equimolecular amounts of sulfuric acid and NH_2 groups are set free, at least until 30 per cent of the nitrogen linkage is hydrolyzed.

Consequently, in heparin, sulfuric acid replaces the acetic acid of the other mucopolysaccharides. The theory that sulfuric acid constitutes the prosthetic group of heparin is thereby still further substantiated.

SUMMARY

In accordance with earlier findings of Wolfrom *et al.*, purified heparin was found to contain only a trace of acetic acid considered to arise from impurities, when analyzed by different methods which, when applied to mucopolysaccharides, give good yields.

The amino group of glucosamine in heparin is not linked to any aldehyde group.

When heparin is boiled in N NaOH, it depolymerizes and loses 75 per cent its anticoagulant activity in 5 minutes. Very little NH_2 nitrogen appears even after 1 hour. If this heparin is subsequently submitted to weak acid hydrolysis in 0.04 N HCl, free sulfuric acid appears at the same time and with the same rate as the NH_2 groups.

Consequently, in heparin a sulfuric acid group is bound to the amino group with a substituted amidosulfuric acid, $—NHSO_2OH$, as the most probable type of linkage.

The authors are greatly indebted to Mr. Gunnar Lindén, Apoteksvarucentralen Vitrum, for his assistance during the performance of the work.

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A STUDY OF THE RATE OF OXIDATION OF THE METHYL GROUP OF DIETARY METHIONINE*

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We have recently reported that the administration *per os* to a rat of a single 200 mg. dose of methionine labeled with C^{14} in the methyl group was followed by the appearance of one-quarter of the radioactive carbon in the expired carbon dioxide in 24 hours (1). This finding clearly demonstrated the ability of the animal organism to oxidize the biologically labile methyl group to carbon dioxide and water. It also posed the question as to the extent of oxidation of the smaller amounts of methionine methyl that are ingested in one feeding as a part of an average diet. Accordingly, a series of experiments was carried out in an attempt to answer this question.

The amino acid diets employed contained either 0.6 or 1.2 per cent methionine, levels of methionine equivalent to those provided by rations containing 17 and 34 per cent casein. These methionine levels covered the range most frequently encountered in adequate diets of both the purified and natural food types. Both of the diets contained 0.2 per cent choline chloride and 0.4 per cent cystine.

In studying the rate of oxidation of a dietary component in the whole animal with the use of isotopic carbon, it is desirable to determine not only the total amount oxidized during a given period of time, but also the actual changes in the rate of oxidation that occur within the same period, for the sharpness with which the oxidation curve rises and falls reflects the rapidity with which the animal is absorbing the exogenous material, destroying or excreting it, and integrating it with its endogenous counterparts already present in the body. When the experimental animal is given free access to the diet, the shape of the oxidation curve is difficult to interpret, since the amount of isotopic food ingested is not known or controlled for any time interval.

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Consequently, in the present study a single 2 gm. portion of the diet containing the radiomethionine was administered by stomach tube to rats which had free access, both before and after this treatment, to the same diet containing ordinary methionine. By determining the amount of radiocarbon dioxide in the expired air collected during hourly intervals following the ingestion of the "radioactive meal," the rise and fall in the rate of oxidation of the methyl group was obtained. The size of the "radioactive meal," approximately one-fifth of the daily food consumption, was dictated by the quantity of radiomethionine required to produce a concentration of radiocarbon dioxide in the expired air that could be measured accurately in the rats receiving the 0.6 per cent methionine diet.

In addition to measuring the per cent of the ingested methyl group converted to carbon dioxide and water, the amount of isotopic methyl carbon excreted in the urine and feces in 24 hours was determined. It was thus possible to compare the 24 hour elimination and retention of exogenous methyl carbon on the high and low methionine diets.

Materials and Methods

The L-methionine labeled with C^{14} in the methyl group that was used in these experiments had been previously synthesized in this laboratory by Melville, Rachele, and Keller (2). The barium carbonate prepared from 1 mg. of methionine gave approximately 125,000 c.p.m. when corrected for background and self-absorption.

The metabolism apparatus for the collection of expired CO_2, urine, and feces and the methods for determining the C^{14} content of these materials have been described in an earlier publication (1). Throughout this paper the C^{14} content of the urine, feces, and expired CO_2 is expressed as a per cent of the ingested methionine, methyl group, or C^{14}. Numerically, all three of these expressions are identical.

The composition of the 0.6 per cent methionine diet is shown in Table I. The 1.2 per cent methionine diet was the same except for the increase in methionine and the corresponding decrease in sucrose. The adequacy of the low methionine diet with respect to labile methyl groups and sulfur-containing amino acids was shown by the fact that male rats, weighing 130 gm., gained as rapidly in a 21 day period on this diet as on the 1.2 per cent methionine diet. At the end of this time the fat content of their livers was approximately 4 per cent.

In the metabolism experiments, young male rats, obtained from Rockland Farms, New City, New York, were fed one of the experimental diets containing non-radioactive DL-methionine for a period of 6 to 10 days. When an animal weighed approximately 150 gm., it was given by stomach tube, at 9.45 in the morning, a single 2 gm. portion of diet in which the ordinary methionine was replaced with L-methionine containing C^{14} in the methyl group.

The procedure employed in administering the radiomethionine diet was as follows. 400 mg. of the mixed fats (Covo and corn oil, Table I), previously chilled in the refrigerator, were fed to the rat on a small spatula. Next, a stomach tube, consisting of a piece of x-ray catheter with a hypodermic needle inserted in the proximal end, was introduced, and the non-lipide part of the diet (less methionine) suspended in 1.5 ml. of water was fed through the tube. This was followed immediately by the administration from a 1 ml. syringe of the radiomethionine (12 or 24 mg.) dissolved in 0.5 ml. of water. Finally, 0.5 ml. of water that had been used to wash out the methionine weighing bottle was given through the stomach tube. About 1 minute was required to give the non-lipide portion of the diet plus methionine.

TABLE I

Composition of 0.6 Per Cent Methionine Diet

	per cent
Sucrose	54.8
Covo*	19.0
Corn oil	1.0
Salt mixture†	4.0
Amino acid mixture‡	20.0
Methionine	0.6
L-Cystine	0.4
Choline chloride	0.2
Vitamins§	

* A hydrogenated vegetable oil.

† Osborne-Mendel salt mixture, No. 1 (3), Eimer and Amend, New York.

‡ The proportion of individual amino acids per 185 parts of mixture is as follows: glycine 1, hydroxy-L-proline 1, L-proline 2, serine 2, L-aspartic acid 2, alanine 4, L-tryptophan 4, L-arginine hydrochloride 6, L-histidine·HCl·H_2O 7, L-tyrosine 10, threonine 14, phenylalanine 15, isoleucine 18, valine 20, L-glutamic acid 20, leucine 26, L-lysine hydrochloride 19, $NaHCO_3$ 14.

§ The composition of the vitamin mixture in mg. or units per kilo of diet is as follows: thiamine hydrochloride 10, riboflavin 10, pyridoxine hydrochloride 10, nicotinic acid 10, calcium pantothenate 50, inositol 100, *p*-aminobenzoic acid 10, folic acid 1, biotin 0.1, α-tocopherol acetate 40, 2-methyl-1,4-naphthoquinone 1, vitamin A 7200 units, vitamin D 1200 units.

After the stomach tube had been withdrawn, the animal was transferred at once to the metabolism apparatus and the urine, feces, and expired carbon dioxide were collected for the next 30 hours. During this time the rat had continuous access to the diet containing non-radioactive methionine. No food was eaten during the day. Food consumption during the night was normal when the 2 gm. that had been given by stomach tube were taken into consideration.

RESULTS AND DISCUSSION

In the experiments reported in this paper, two rats were fed the 0.6 per cent methionine diet and four rats the 1.2 per cent methionine diet. In every case the oxidation of the methyl group of the labeled methionine began soon after the ingestion of the diet, as shown by the presence of C^{14} in the carbon dioxide collected during the 1st hour. In two of the four animals receiving the high level of methionine the rate of oxidation reached its maximum in the 2nd hour and declined slightly during the 3rd hour. The other rats on this diet, as well as the two animals on the low methionine diet, attained their respective maximum rates of oxidation of the methyl group during the 3rd hour. After the 3rd hour the rate of oxidation in every animal declined sharply. Low and relatively constant rates of oxidation were reached at the 7th hour by the animals on the 0.6 per cent methionine diet, and at the 7th or 8th hour by the rats on the 1.2 per cent diet.

The average curves showing the rate of oxidation of the methyl groups of dietary methionine to carbon dioxide[1] and water by the two sets of rats are given in Fig. 1. While differing considerably in magnitude, they possess the same general shape, and it is the significance of their shape with respect to the metabolism of the ingested methyl group that will be first discussed.

As indicated by the oxidation curves, the metabolism of the methyl group of dietary methionine falls into two phases; the first is characterized by a rapid rise and fall in the rate of oxidation, and the second by the establishment of a relatively steady state. For convenience the descriptive terms "period of assimilation" and "period of equilibrium" may be used to indicate these two phases. In the period of assimilation the rise in the rate of oxidation is a measure of the rate of absorption of the exogenous methionine and of the concentration of the tagged methyl group at the active centers of oxidation. The almost equally rapid decline in the rate of oxidation after the 3rd hour reflects a fall in the concentration of radiomethyl at the active centers of oxidation.[2] While the process of oxidation will in itself lower the concentration, earlier observations on the chemical and anatomical distribution in the body of the methyl group administered as methionine (1) suggest that concurrent *chemical* and *anatomical* translocations of the methyl group also contribute to the decline in the rate of oxidation.

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[1] The sensitivity with which the oxidation curves of the methyl group mirror the actual rates of conversion of labeled methyl radicals to CO_2 in the body is indicated by the report of Gould and coworkers (4) that the maximum concentration of $C^{14}O_2$ in the expired air occurs within 10 minutes after the injection of radioactive bicarbonate. The figures given in the present paper for the oxidation of the methyl group have not been corrected for the $C^{14}O_2$ that was formed but subsequently entered into synthetic reactions in the body. According to Gould and coworkers (4) over 90 per cent of an injected dose of bicarbonate is eliminated as respiratory CO_2 in 4 hours. Since in our experiments the urine was collected in acid, any $C^{14}O_2$ excreted as bicarbonate was collected along with the respiratory $C^{14}O_2$.

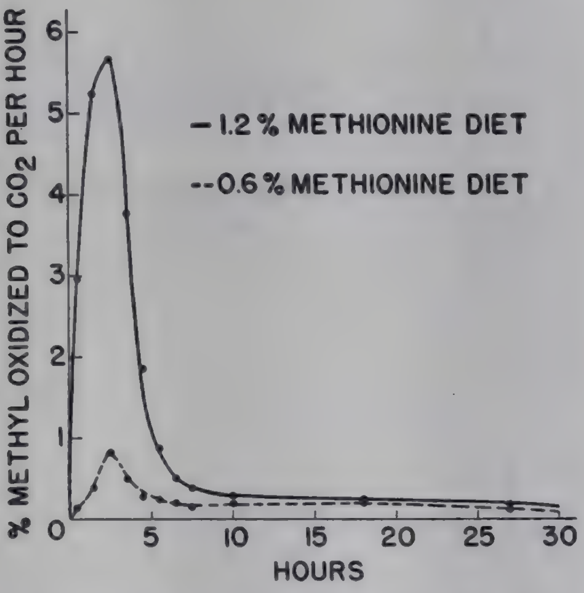

FIG. 1. The rate of oxidation to CO_2 of the methyl group of methionine ingested in a single 2 gm. portion of diet. The diets containing L-methionine labeled with C^{14} in the methyl group were administered by stomach tube to 150 gm. male rats which, both before and after this treatment, had continuous access to the corresponding diets containing ordinary methionine. The oxidation curve for the 0.6 per cent methionine diet is an average of two experiments and the curve for the 1.2 per cent methionine diet is an average of four experiments.

The period of equilibrium was initiated by the establishment of a low and relatively constant rate of oxidation. This indicates that the *major* (most rapid) chemical conversions and anatomical translocations of the radiomethyl group have occurred, and that it and its oxidation products[3] have merged with their counterparts already present in the body. However, as we have previously shown (1), the concentration of the ingested methyl carbon in the tissues and organs is far from uniform even after 52 hours. Presumably a continuous slow redistribution of the radiomethyl group (and its oxidation products) follows this initial rapid but uneven distribution.

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[2] *In vitro* studies in this laboratory have shown that the methyl group provided by methionine is oxidized by liver and kidney cortex, but not by heart muscle or testes. There is at present no evidence to indicate whether the initial step in the oxidation can occur while the methyl group is attached to the S of methionine, or whether it takes place only after the methyl group has been transferred to some other molecule.

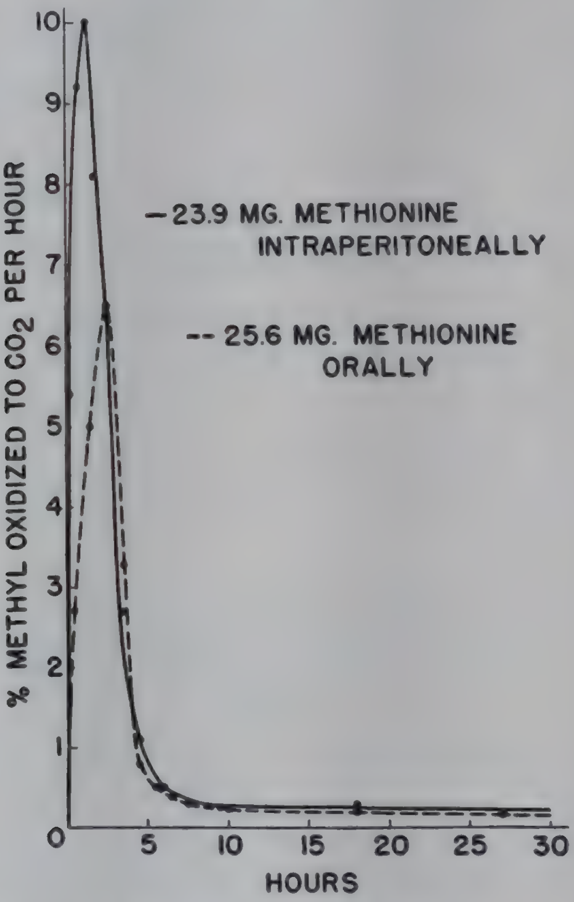

FIG. 2. The effect of oral and intraperitoneal administration on the rate of oxidation to CO_2 of the methyl group of methionine labeled with C_{14} in the methyl radical. The oral dose was given by stomach tube as a component of 2 gm. of a 1.2 per cent methionine diet. The injected dose was given immediately after the administration of 2 gm. of a methionine-free diet. Before and after treatment both animals (150 gm. male rats) had continuous access to a diet containing 1.2 per cent of ordinary methionine.

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[3] We have isolated radioformaldehyde from liver homogenates incubated with sarcosine labeled with C^{14} in the methyl group (5). Moreover, as we reported at the meeting of the American Society of Biological Chemists at Detroit, 1949, the presence of radioactive formic acid has been indicated by the evolution of $C^{14}O_2$ when the distillate from the formaldehyde-free homogenate is oxidized with $HgCl_2$.

In view of the prompt and rapid rise of oxidation during the period of assimilation, the possibility that the intestinal flora contributed significantly to the initial degradation of the exogenous methyl group was investigated. A rat on the 1.2 per cent methionine diet was fed 2 gm. of the ration without methionine and then injected intraperitoneally with 23.9 mg. of radiomethionine. The oxidation curve is shown in Fig. 2. The curve for Rat 3501 (see also Table II), fed 25.6 mg. of radiomethionine in the diet, is also shown. In so far as can be ascertained by comparing the results of oral and parenteral administration, the intestinal bacteria did not participate extensively in the initial oxidation (or the 24 hour oxidation) of the methyl groups.

TABLE II

Oxidation and Excretion of Methyl Group of Dietary Methionine

The experimental diet contained 0.4 per cent cystine, 0.2 per cent choline chloride, and 0.6 or 1.2 per cent methionine. Male rats (150 gm.) were fed by stomach tube a single portion of diet in which L-methionine labeled with C^{14} in the methyl group replaced the ordinary methionine otherwise employed. The oxidation and excretion of the radiomethyl carbon was measured for 24 hours.

Rat No.	Radioactive methionine ingested	Per cent C^{14} oxidized to $C^{14}O_2$				Per cent C^{14} excreted*		Per cent C^{14} eliminated	C^{14} retained†
		0–6 hrs.	6–12 hrs.	12–24 hrs.	Total (A)	Urine (B)	Feces (C)	(A+B+C)	
	mg.								*mg.*
3498	11.9	1.5	1.0	2.6	5.1	3.70	0.32	9.12	10.8
3592	12.3	3.2	1.2	2.1	6.5	5.04	0.31	11.85	10.8
3500	24.2	23.4	2.1	3.3	28.8				
3501	25.6	18.8	1.6	2.3	22.7	4.02	0.29	27.01	18.7
3507	24.4	17.6	2.4	3.3	23.3	4.71	0.50	28.51	17.5
389	25.3	21.6	2.0	2.8	26.4	4.71	0.34	31.45	17.3
3497‡	23.9	27.1	1.8	3.4	32.3	4.56			

* Not all of the excreted C^{14} is present as methyl groups; a portion represents their oxidation products.

† Expressed as mg. of methionine.

‡ In this rat the radiomethionine was injected intraperitoneally immediately after the administration by stomach tube of 2 gm. of diet from which methionine was omitted.

The methionine content of the diet had a profound effect on the amount of dietary methyl groups converted to carbon dioxide. On the low methionine diet 6 per cent and on the high methionine diet 25 per cent of the ingested methyl carbon appeared in the expired air as radioactive carbon dioxide in 24 hours. This increase in the proportion of dietary methyl groups oxidized daily was accomplished almost entirely by a great increase in the rate of oxidation during the period of assimilation, as is shown in Table II. Doubling the methionine content increased the rate of oxidation approximately 9-fold during the first 6 hours. On the other hand, the increase in the rate of oxidation during the period of equilibrium was relatively slight.

The relation between the previous methionine intake and the high rate of oxidation in the period of assimilation on the 1.2 per cent methionine diet was investigated. A rat which had been maintained on the 0.6 per cent methionine regimen was given a 2 gm. portion of the 1.2 per cent radiomethionine diet by stomach tube and then returned to the lower level of methionine. The rate of oxidation (Rat 389, Table II) was just as high in this animal as in those maintained on 1.2 per cent methionine for 7 days before treatment. Consequently, the ability to oxidize the methyl groups at a faster rate on the higher level of methionine was not due to an adaptive increase in the concentration of the oxidative enzymes.

Probably the ingestion of the 24 mg. dose of radiomethionine as contrasted to the 12 mg. dose resulted in the saturation of the synthetic enzymes, thus increasing the proportion of methyl groups available for destruction by the oxidative enzymes. From this point of view, the lower rate of oxidation in rats fed 0.6 per cent methionine was the result of competition for the ingested methyl groups between synthetic and degradative (oxidative) enzyme systems.

An appreciable quantity of the ingested methyl groups was eliminated in the urine in 24 hours in the form of methyl compounds or the oxidation products of methyl groups.[4] On the 0.6 per cent methionine diet the amount of C^{14} present in the urine was nearly as high as the amount of C^{14} present in the expired carbon dioxide (Table II). Much smaller amounts were present in the feces. However, doubling the level of methionine had little effect on the per cent of exogenous methyl carbon excreted in either the urine or feces. This is in contrast with the considerable increase in the rate of oxidation during the period of assimilation produced by doubling the methionine intake, and it further emphasizes the contribution of oxidation, in this phase of metabolism, to the regulation of the methyl balance of the body.

Nevertheless, the higher rate of oxidation of the methyl group on the 1.2 per cent methionine diet fell short of reducing the amount of ingested methyl carbon retained by the body in 24 hours to the level that prevailed on 0.6 per cent methionine. The retention of C^{14} was 10.8 mg. (expressed as methionine) on the low methionine diet and 17.8 mg. on the high methionine diet (Table II).

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[4] One oxidation product is the CO_2 which has been used in the synthesis of urea. The carbon of urea possesses the same specific activity as the carbon of the expired CO_2 (6). Since the amount of urea carbon equals about one-thirty-third of the carbon present in the expired CO_2, the per cent of ingested C^{14} present as urea may be approximated by multiplying the per cent present as expired CO_2 by 0.03.

Since both rations contained an adequate supply of labile methyl groups, and since the animals on both rations gained at the same rate, it does not seem likely that the increased retention of C^{14} was due to an over-all increase in the storage of methyl groups or their oxidation products. More probably, the ingestion of the high methionine diet increased the oxidation and excretion of non-radioactive methyl groups already present in the body, thus increasing the rate of turnover. Also, raising the methionine level may have increased the oxidation of the methyl groups simultaneously consumed in the form of choline. In either event the retention of newly ingested methyl groups would be increased without appreciably changing the concentration of total methyl groups in the body.

SUMMARY

The rate of oxidation of the methyl group of dietary methionine has been measured in rats fed diets containing choline and cystine, and either 0.6 or 1.2 per cent methionine. This was accomplished by determining the amount of radiocarbon dioxide expired following the ingestion of a single meal in which C^{14}-labeled methionine replaced the ordinary methionine otherwise present in the diet.

On both diets the oxidation of the radiomethyl group fell into two phases. During the first phase, or period of assimilation, there was a rapid rise in the rate of oxidation to a maximum attained during the 3rd hour, followed by a decline until the 7th or 8th hour. The second phase, or period of equilibrium, was initiated at this time by the establishment of a low and relatively constant rate of oxidation.

Doubling the methionine content of the diet produced a 9-fold increase in the per cent of radiomethyl groups oxidized during the period of assimilation. It had relatively little effect on the rate of oxidation during the period of equilibrium, or on the per cent of radiomethyl carbon excreted in the urine or feces in 24 hours.

The implications of these results are discussed.

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THE ORIGIN OF THE METHYL GROUP OF EPINEPHRINE*

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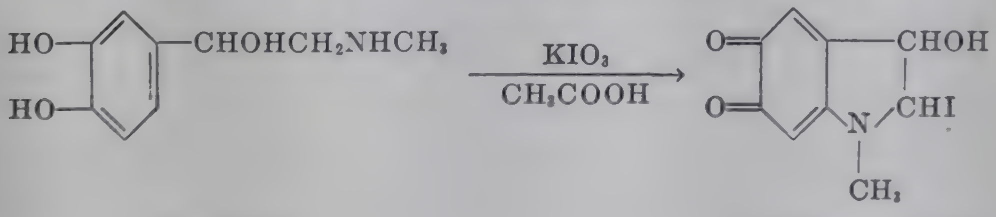
In the original communication on the effect of choline upon the growth of the rat on a cystine-methionine-free diet supplemented with homocystine (1), it was suggested that, "The methionine-homocystine-choline relationship may be significant, not only to sulfur and fat metabolism, but also to other problems of methylation in the animal organism." Later, when the transfer of methyl groups from methionine to choline and creatine had been demonstrated by the crucial experiment in which deuteriocholine and deuteriocreatine were isolated from the tissues after the feeding of deuteriomethionine to the rat (2), the possibility was raised that the various *N*-methyl and *S*-methyl compounds synthesized in the body might derive their methyl groups from the dietary methionine, choline, and betaine.

Our first opportunity to test this hypothesis was afforded by some experiments with the rabbit (3). In addition to choline and creatine, anserine was isolated after deuteriomethionine had been fed. It was found that the methyl group of the isolated anserine was derived from the ingested methionine.

In the present paper this hypothesis has been tested further by an investigation of the origin of the methyl group of epinephrine. This work was made possible by the availability of methionine containing radiocarbon



Ephinephrine

Iodoadrenochrome

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in the methyl group (4). The radiomethionine was administered to rats and the epinephrine was isolated from the adrenals with the aid of "carrier" epinephrine. It was found that the epinephrine contained a significant amount of radiocarbon. The specific activity of the epinephrine isolated remained constant on recrystallization. The recrystallized ephinephrine was then converted to iodoadrenochrome (5), a quinonoid compound differing markedly from epinephrine in solubility. The specific activity of the iodoadrenochrome was the same as that of the epinephrine within the limits of experimental error.

EXPERIMENTAL

Administration of Methionine Containing C^{14} in Methyl Group—Two separate experiments were carried out on two adult rats. The choline-free diet had essentially the same composition as in the experiment of Keller, Rachele, and du Vigneaud (6). The feeding experiments with radioactive methionine were preceded by 3 days feeding of ordinary methionine in order to accustom the rats to the diet. For the duration of the experiment the animals showed the normal gain in weight. The first rat received over a period of 4 days at a level of 1 per cent in the diet 593 mg. of radioactive L-methionine with a radioactivity of 6.49×10^6 c.p.m. per mM. The second rat received over a period of 4 days at a level of 0.6 per cent in the diet 360 mg. of radioactive L-methionine with a radioactivity of 4.22×10^7 c.p.m. per mM. In the case of the second rat, the diet for the pre-experimental period contained 0.6 per cent methionine. The radioactive methionine was prepared by the method of Melville, Rachele, and Keller (4).[1]

Isolation of Epinephrine from Adrenals; Recrystallization; Conversion to Iodoadrenochrome—In each experiment the rat was killed with chloroform at the end of the feeding period. The adrenal glands were removed, ground rapidly with sand in a small sintered glass filter, and extracted with six 0.25 ml. portions of cold 5 per cent trichloroacetic acid, each portion being filtered off with gentle suction. To the trichloroacetic acid extract were added 25.0 mg. of *dl*-epinephrine as a carrier, and a few crystals of sodium sulfite to prevent oxidation of the epinephrine by atmospheric oxygen. The solution was then extracted with five 1.5 ml. portions of peroxide-free ether in order to remove the bulk of the trichloroacetic acid. The solution was filtered and an excess of ammonia was added to the filtrate. The solution was then chilled in an ice bath. The epinephrine which crystallized out was filtered onto a weighed filter paper with an

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[1] The $BaC^{14}O_3$ used as starting material was supplied by the Monsanto Chemical Company, Clinton Laboratories, on allocation from the United States Atomic Energy Commission.

Allihn type filter (7), washed with alcohol, dried, and weighed. All the samples of epinephrine and iodoadrenochrome were prepared in this way for counting, as a mat of fine crystals having an area of 2.32 sq. cm. and weighing 4 to 9 mg. per sq. cm.

After the radioactivity of the epinephrine was determined by the method described in the next section, it was dissolved off the filter paper with 5 ml. of 0.1 per cent acetic acid. A few crystals of sodium sulfite were added, the solution was filtered, and the epinephrine was recrystallized by adding ammonia. The radioactivity of the recrystallized material was determined.

The recrystallized epinephrine was then converted to iodoadrenochrome by the method of Richter and Blaschko (5). According to these workers and Bergel and Morrison (8), the structure of this compound is indicated to be 2-iodo-3-hydroxy-1-methyl-2,3-dihydroindole-5,6-quinone.

To convert the epinephrine to iodoadrenochrome, it was dissolved in 0.2 ml. of 2 per cent acetic acid, and 0.4 ml. of 4 per cent potassium iodate was added. The dark red crystals of iodoadrenochrome which crystallized out of solution were filtered, washed with water and alcohol, dried, weighed, and analyzed for radiocarbon. In the two experiments 10.3 mg. and 6.7 mg. of iodoadrenochrome were obtained. These amounts are equivalent to 6.2 mg. and 4.0 mg. of epinephrine, respectively.

Radiocarbon Analyses—The radiocarbon analysis on the radiomethionine was carried out by the method described by Keller, Rachele, and du Vigneaud (6). Briefly, this involves the burning of a weighed sample of the radiomethionine, the collection of the CO_2 in alkali, and precipitation of it as $BaCO_3$ from an aliquot of the alkaline solution. The $BaCO_3$ is then filtered onto a weighed filter paper, washed, dried, and weighed. The same filtration apparatus (7) was used for the $BaCO_3$, epinephrine, and iodoadrenochrome samples.

Each sample of epinephrine or iodoadrenochrome was counted, along with the $BaCO_3$ sample from the radiomethionine, with a thin mica window bell-shaped Geiger-Müller counter and scaling circuit. The activity of each sample was corrected for background and then for self-absorption by using the theoretical self-absorption equation (9), and a value of the absorption coefficient of 0.32 sq. cm. per mg. (10). The specific radioactivities expressed in counts per minute per mM are given in Table I. The over-all error involved in the measurements and calculations was ±8 per cent.

Epinephrine Content of Adrenals—Because of the small amount of epinephrine present in the adrenals of the rat, it was not possible to determine the amount present in the above isolation experiments. However, some preliminary experiments were carried out to determine the approximate amount of epinephrine in the adrenals of adult rats having about the same weight as those used for the isolation experiments. The epinephrine was extracted from the adrenals by the procedure described above, and the extracts were analyzed for epinephrine by the colorimetric method of Schild (11). This method depends on the oxidation of epinephrine to a red quinone by iodine and has been found to be a reliable colorimetric method for determining epinephrine. The average of two values obtained for the epinephrine in the combined adrenals of an adult rat was 30 γ.

Table I

Radioactivity of Epinephrine Isolated from Adrenals of Rats Fed Methionine Containing C^{14} in Methyl Group

Rat No.	Compound	Weight	Radioactivity
		mg.	*c.p.m. per mM*
I (250 gm.)	Radioactive methionine fed	593	6.49×10^6
	Epinephrine and carrier, isolated	20.6	9.34×10^2
	" " " recrystallized	9.3	9.20×10^2
	Iodoadrenochrome	10.3	9.77×10^2
II (187 gm.)	Radioactive methionine fed	360	4.22×10^7
	Epinephrine and carrier, isolated	19.2	4.34×10^3
	" " " recrystallized	10.7	4.27×10^3
	Iodoadrenochrome	6.7	4.47×10^3

RESULTS AND DISCUSSION

The constancy of the specific activity of the epinephrine on recrystallization and preparation of a derivative indicates that the radioactivity was incorporated in the epinephrine and not present as an impurity. This point was, of course, quite essential in this experiment, since the amount of epinephrine present in the adrenals is so minute. The derivative selected, iodoadrenochrome, is much less basic than epinephrine, crystallizing out of acetic acid solution. If the activity of the epinephrine had been due to an impurity, it is highly improbable that this radioactive impurity could have been carried down by the iodoadrenochrome crystals in the same molar amount.

In the first experiment in which methionine containing C^{14} in the methyl group was fed to a rat it was found that the methyl group of methionine can be oxidized to carbon dioxide *in vivo* (12). In the experiment on the first rat described in this paper the radioactivity of the expired carbon dioxide was also measured during the 4 days of feeding the radiomethionine. It was then possible to compare the specific activity of this carbon dioxide with that of the methyl group of the epinephrine on the assumption of an epinephrine content of approximately 30 γ in the adrenals of this rat.

The specific activity of the methyl group of the epinephrine was enormously greater than that of the expired carbon dioxide. From this result we concluded that the radiocarbon in the epinephrine could not have been derived from carbon dioxide and that it must have been introduced into the epinephrine by a transmethylation reaction. We cannot say from these data, however, whether methionine is the immediate precursor of the methyl group of epinephrine or whether an intermediate methyl donor is involved.

The fact that the unmethylated portion of the epinephrine molecule can be derived *in vivo* from phenylalanine has been established by Gurin and Delluva (13), who injected rats with phenylalanine containing C^{14} in the carboxyl group and the α position and isolated epinephrine containing C^{14} from the adrenals by the carrier technique. By a degradation reaction they were able to show that all the C^{14} was in the carbon corresponding to the α-carbon of phenylalanine.

The work of Gurin and Delluva and the present work therefore point to the two essential amino acids phenylalanine and methionine as precursors of epinephrine. The present interest in the interrelationship of arterenol and epinephrine makes the involvement of transmethylation in the biosynthesis of epinephrine all the more intriguing.

SUMMARY

Epinephrine has been isolated by a "washing out" technique from the adrenals of two rats which received methionine containing C^{14} in the methyl group. The epinephrine was found to contain a significant amount of C^{14}. That this C^{14} was incorporated in the epinephrine and not present as an impurity was shown by the fact that the specific activity of the epinephrine remained constant on recrystallization and on the subsequent conversion of the epinephrine to iodoadrenochrome.

It has been concluded from these results that the methyl group of epinephrine can be derived from dietary methionine *in vivo*.

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THE COMPOSITION OF CERTAIN FRACTIONS OBTAINED BY THE ACTION OF RIBONUCLEASE ON RIBONUCLEIC ACID FROM YEAST

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The isolation of a homogeneous preparation of a nucleic acid is yet to be reported. However, sufficient information on purified heterogeneous preparations has accumulated during the last decade to indicate that ultimately a number of structurally different nucleic acids may be found. The largest unit which at present is recognized as a well characterized constituent of the nucleic acids is the mononucleotide. Precise information concerning the order and mode of arrangement of the mononucleotides in the nucleic acids is lacking. Seemingly then to bridge the gap between the mononucleotide and the macromolecular nucleic acids, the isolation and characterization of polynucleotide fragments of nucleic acids become obligate.

At present the only easily controlled fragmentation of ribose nucleic acids is that which is catalyzed by the enzyme ribonuclease. Apart from the work of Loring and Carpenter (1), who have reported that mononucleotides may be isolated from the products of the action of ribonuclease upon a preparation of ribonucleic acid from yeast, it is commonly assumed from the prior work of others (2–5) that the fragments which result from such action are largely polynucleotide in character.

Owing to similarities in their chemical and physical properties the fractionation of mixtures of polynucleotides presents a difficult task. An initial gross separation of the products resulting from ribonuclease action may be brought about by the use of dialysis. The formation of an acid-insoluble, non-dialyzable fraction as a result of the action of ribonuclease upon ribonucleic acid was first noted by Schmidt and Levene (2) and later confirmed by Kunitz (3). Fischer, Böttger, and Lehmann-Echternacht (5) subjected purified, dialyzed ribonucleic acid to the action of ribonuclease, dialyzed the digest, and calculated the average molecular weight for the components present in the dialyzable solute to be 690. Loring, Carpenter, and Roll (6) dialyzed the reaction mixture and recovered the non-dialyzable residue in two fractions. One fraction was obtained by the use of glacial acetic acid as a precipitant, while the other fraction was obtained by the use of alcohol. Tests for solubility or homogeneity of the fractions which were obtained in this manner were not reported.

Certain published (7) and unpublished experiments conducted in this laboratory, in which the foregoing techniques or combinations of techniques were used, gave results which varied according to the time of dialysis and manner of precipitation of the solute from the digestion mixture. Consideration of the varied results led to the conclusion that the dialyzable fractions which result from the action of ribonuclease are not of constant composition and might contain labile linkages susceptible to chemical separation. Thus in order that successive dialysates and ultimately the residue should represent so far as possible the successive products of enzymic action, an experiment was devised in which reactions were carried out in the cold, dialysates were collected after short intervals of time, and the solute was recovered by lyophilization.

EXPERIMENTAL

A commercial sample of ribonucleic acid from yeast (Schwarz) was purified by the procedure described by Levene and Bass (8) with the additional steps of deproteinization by the method of Sevag, Lackman, and Smolens (9). The final product in the form of its sodium salt at pH 7.0 was dialyzed against distilled water and recovered by lyophilization.

15 gm. of this ribose nucleic acid were dissolved in 450 ml. of boiled, distilled water which had been cooled to 10°. The pH was adjusted to 7.0 by the use of a dilute solution of sodium hydroxide. 45 mg. of crystalline ribonuclease (Armour and Company) were added. The solution was placed in dialysis tubing (Visking sausage casing) with a flat width of $1\frac{3}{8}$ inches. The tubing was covered with cold, boiled, distilled water and placed in a cold room at approximately 5°. At the intervals given in Table I the dialysate was removed and replaced by cold, boiled, distilled water. In each case the dialysate was immediately lyophilized. Near the conclusion of dialysis the residue had become considerably diluted by osmosis. The level of the interior solution during the formation of Fraction 5 of the dialysate rose above the level of the exterior solution. In the collection of the remaining fractions the interior solution was kept submerged. Since it was considered that the dilution might prevent complete dialysis of dialyzable material, the experiment was concluded by lyophilizing the residue after collection of Fraction 10 of the dialysate. A 2.0 gm. sample of this residue was dissolved in 50 ml. of water. The pH was adjusted to 7.0 and dialysis was continued for 4 more days. Fraction 11 of the dialyzable material and residue were lyophilized and recovered. A control of 3.0 gm. of the original ribonucleic acid was dialyzed in parallel with the experiment. The total dialysate and residue from the control were recovered by lyophilization.

Total nitrogen was determined by duplicate micro-Kjeldahl analyses.

TABLE I

Composition of Dialysates, Residues, and Controls Obtained by Action of Ribonuclease on Ribonucleic Acid from Yeast

Dialysate fraction No.	1	2	3	4	5	6	7	8	9	10	11	Residue	Control dialysate	Control residue
Dialysis time per fraction, *hrs.*	2.5	6	11	25	9.5	19	23	24	22.5	27	96		169	
Weight of fractions, *gm.*	1.3	1.8	1.6	1.8	0.4	0.8	0.3	0.2	0.2	0.4	0.8*	1.3*	0.2	2.5
Total N, %	13.14	13.28	13.41	14.12	14.43	13.01	13.24	13.32	14.30	14.90	13.70	15.40	11.40	13.90
$\frac{\text{Guanine N}}{\text{Total N}}$, %	29	28	31	35	35	35	38	38	40	41	35	47	34	35
$\frac{\text{Purine N}}{\text{Total N}}$, %	60	59	62	70	72	69	70	72	74	79	75	82	72	67
Total P, %	8.48	8.69	8.70	8.51	8.35	7.73	7.84	7.74	7.84	8.08	7.89	8.14	6.68	8.37
$\frac{\text{Labile P}}{\text{Total P}}$, %	41	40	42	51	55	51	52	55	60	64	58	69	54	51
$\frac{\text{Total N}}{\text{Total P}}$	1.55	1.53	1.54	1.66	1.73	1.68	1.69	1.72	1.82	1.84	1.74	1.89	1.71	1.66
$\frac{\text{Non-purine N}}{\text{Stable P}}$, *atoms*	2.3	2.3	2.2	2.2	2.4	2.4	2.3	2.4	2.6	2.4	2.3		2.3	2.5
% soluble in uranyl reagent	51	53	47	34	26	33	32	27	11	10	27		7	

* Residue which remained after Fraction 10 was 3.4 gm. A 2.0 gm. sample of this residue was dialyzed to obtain Fraction 11 (see the text).

Statistical analysis of the data for total nitrogen indicated that 95 per cent of the means of the duplicate analyses were within 0.7 per cent of the true value.

The content of total purine nitrogen was determined by the use of the method of Kerr and Seraidarian (10) which was modified in certain minor details. The amount of copper sulfate which was used was three-quarters of the recommended amount. Hydrolysis was conducted in an N solution of hydrochloric acid for 45 minutes in a boiling water bath. The method was tested on three samples which contained 0.665 mg. of guanine nitrogen in the presence of 2 moles of cytidylic acid per mole of guanine; the average recovery of guanine nitrogen was 102 per cent. The average recovery of pure guanine from solutions in which guanine was the only solute was 97 per cent.

After the isolation of purines in duplicate by the method of Hitchings and Fiske (11), guanine was determined in triplicate by the colorimetric procedure described by Hitchings (12). Analysis of the data indicated that 95 per cent of the means of duplicate isolations are within 3 per cent of the true value. At this point a word of caution regarding the direct application of Hitchings' method to the hydrolysates of nucleic acids or derivatives may be in order. Loring and coworkers (6) have stated that "guanine is the only compound known to be present in nucleic acid that gives a positive reaction." However, under the conditions that are necessary to hydrolyze purines from their derivatives, furfural will form from free ribose. Furfural reacts with the phenol reagent. Comparison experiments performed in this laboratory have yielded high results for guanine when Hitchings' procedure has been applied directly to hydrolysates of nucleic acids and of purine nucleosides.

The partition of phosphate was performed as described by Bacher and Allen (13). Analyses for the content of purines and labile phosphate have been performed on a number of samples. In the most highly purified samples of nucleic acids and in those fractions which were derived by the action of ribonuclease, the labile phosphate corresponded, within 3 per cent, to the content of purines. However, the dialysates from partially purified nucleic acids, certain samples of partially purified nucleic acids, and one highly purified sample which had been in storage for 7 years did not give quantitative correspondence between labile phosphate and content of purines.

DISCUSSION

The entire experiment was conducted at 5°. The temperature coefficient of the enzyme-catalyzed reaction is much greater than the temperature coefficient of dialysis; hence it could be expected that low temperatures would permit dialysis to parallel the reaction. Thus, successive dialysates should to a certain degree represent the successive products of the enzymic action. The attainment of the objective of the experiment falls short of the ideal, owing to the lack of information concerning many phases of the problem such as (*a*) the nature of the action of ribonuclease, (*b*) the structures of the substrates, and (*c*) the characteristics of membranes toward dialysis of polynucleotides.

In addition to the preparation of ribonucleic acid which is used in the present experiment, two other preparations from two different commercial sources were purified and analyzed. Within the limits of accuracy of the analytical procedures, all of the preparations contain approximately equimolar quantities of the four constituent mononucleotides. Also the control preparation in this experiment, after continued dialysis for over a week and after lyophilization and analysis, contained equimolar quantities of the four constituent mononucleotides. Studies in progress concerning the solubility of these preparations show that the term ribonucleic acid from yeast, at least as applied to these preparations, designates a mixture of at least three components.

According to the analyses of the fractions of the dialysate (Table I), all of the fractions are heterogeneous. Certain trends in the change of composition may be noted. Fractions 1, 2, and 3 are similar in composition. The molar ratio of the purine-containing constituents to the pyrimidine-containing constituents is approximately 2:3. The same molar ratio for the average components of Fractions 4, 5, and 6 is approximately 1:1. The ratio for the remainder of the fractions of the dialysate, though variable, averages 3:2.

The atomic ratio of non-purine nitrogen to stable phosphorus is a measure of the ratio of uracil to cytosine. Thus, the first few dialyzable fractions contain a preponderance of uracil over cytosine. A preliminary experiment gave the same result (7). The later fractions contain approximately equal amounts of the two pyrimidines. Since the non-purine nitrogen is obtained by difference, caution must be exercised in its use. For the data to have significance the substance should have a high content of pyrimidines and must be free from protein. These conditions are met by the dialyzable fractions but not by the non-dialyzable residues.

The first fractions contain a greater proportion of nucleotides of lower molecular weight than do the later fractions. This is shown by the behavior of the fractions toward precipitation with uranyl ion. Zittle's modification (14) of MacFadyen's uranyl reagent (15) was employed. Studies on mononucleotides have shown that they are soluble in the uranyl reagent (14). Nothing is known concerning the solubility of di- and trinucleotides. Polynucleotides of a higher order are considered to be insoluble. Therefore, the decreasing order of solubility, which is noted as enzymic action and dialysis proceeds, can be taken as a qualitative measure of the size of the polynucleotides in the fractions. If it is assumed that all of the components which are soluble in the presence of uranyl ion consist of mononucleotides, then approximately one-third of the total dialyzable fraction comprises mononucleotides. Also about two-thirds of the material which might conceivably be in the nature of mononucleotides is formed in the first 20 hours of enzymic action.

Prior work (6, 16, 17) indicates that ribonuclease may catalyze the hydrolysis of a phosphate linkage between certain constituent pyrimidine nucleotides and the adjacent nucleotide. If this assumption is applied, then the first fractions of the dialysate represent predominantly the action of ribonuclease on the part of the nucleic acid molecule, in which the spacing of certain pyrimidine nucleotides is more frequent relative to purine nucleotides than is found with the higher molecular weight components which predominate in the later fractions. Since the non-dialyzable residue, which is polynucleotide in character, contains appreciable quantities of pyrimidines, it should be emphasized that not all of the phosphate linkages between pyrimidine nucleotides and adjacent nucleotides are hydrolyzed by ribonuclease. The specificity of ribonuclease probably is such that not only certain specific linkages but also a certain arrangement of groups adjacent to the linkage are necessary for action. The substrate requirements of the peptidases is a well known example of such specificity.

As mentioned earlier, the analysis of highly purified samples of nucleic acids and of those fractions which were isolated as a result of the action of ribonuclease showed a correspondence between the content of purines and labile phosphate. Thus, the action of ribonuclease does not break pyrimidino-ribosidic bonds. If such bonds were broken, the resulting ribose-3-phosphate would have registered as labile, not stable, phosphate (13).

In the present experiment three-fourths of the material is rendered dialyzable by the action of ribonuclease. In experiments on three other samples of ribonucleic acid, to be reported later, the dialyzable fractions were found to be 62, 72, and 80 per cent.

SUMMARY

Ribonucleic acid from yeast was purified and dialyzed, neutralized to pH 7.0, and subjected to the action of ribonuclease at 5°. The dialyzable fractions which resulted from the enzymic action were collected at intervals and immediately recovered by lyophilization. After the completion of enzymic action and of dialysis, the non-dialyzable residue was recovered by lyophilization.

Analyses indicated the original ribonucleic acid to be composed of approximately equimolar quantities of the four constituent mononucleotides. Fractions of the dialysate were found to be heterogeneous. With progressive enzymic action and collection of dialysate the molar ratios of the purine-containing constituents to the pyrimidine-containing constituents passed from 2:3 through 1:1 to 3:2. The corresponding ratio for the non-dialyzable residue was 7:3.

The bearing of these data on the nature of ribonucleic acid and the mode of action of ribonuclease is discussed.

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A COMPARISON OF PENTOSE NUCLEIC ACID FROM PANCREAS WITH RIBONUCLEIC ACID FROM YEAST

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Owing to the relatively high content of guanylic acid (1–5), the pentose nucleic acid from pancreas has always been considered to be distinct from pentose nucleic acids from other sources. It was noted by Eiler and Allen (6) that their preparations of pentose nucleic acid from pancreas behaved similarly to the ribonucleic acid from yeast when subjected to the action of ribonuclease. Later, it was found that the action of ribonuclease on ribonucleic acid from yeast produced an acid-insoluble, non-dialyzable residue which contains a high percentage of guanine (7, 8). The high content of ribonuclease in pancreatic tissue (9–11), the rapid rate of action of ribonuclease, the mild conditions under which the pentose nucleic acid from pancreas is isolated, and the high content of guanine in such preparations suggest that the preparations which have been reported to date may be mixtures of the native nucleic acid and the more insoluble fragments.

Initial advances in the study of the nature of the native pentose nucleic acid from pancreas may be attained from investigations which relate to (*a*) the isolation of the nucleic acid by mild procedures which block action by ribonuclease, and (*b*) comparisons of the non-dialyzable residues which remain after the action of ribonuclease on the pentose nucleic acids from pancreas and from other sources.

EXPERIMENTAL

Four preparations of pentose nucleic acid from pancreas were isolated. The first two preparations were obtained by following the procedure outlined by Jorpes (1, 2), with the additional steps of final deproteinization by the method of Sevag, Lackman, and Smolens (12). The first isolation was made from commercial frozen pancreas glands. Analyses according to methods described in previous publications (8, 13) yielded the following results: total nitrogen 15.90 per cent, ratio of guanine nitrogen to total nitrogen 0.57, ratio of purine nitrogen to total nitrogen 0.75, total phosphorus 8.27 per cent, ratio of labile phosphate to total phosphate 0.67, ratio of nitrogen to phosphorus 1.92.

For the second isolation, pancreatic glands from cattle were sliced and packed in dry ice immediately upon extirpation. The frozen glands were worked up in small batches so that every possible application of speed and low temperature could be made. Both the crude and the finally purified nucleoproteins which were isolated as a part of the procedure gave qualitative tests for ribonuclease activity (9). Analyses gave results similar to those obtained for the first preparation: total nitrogen 16.67 per cent, ratio of guanine nitrogen to total nitrogen 0.54, ratio of purine nitrogen to total nitrogen 0.80, total phosphorus 8.83 per cent, ratio of labile phosphate to total phosphate 0.67, ratio of nitrogen to phosphorus 1.89.

The third isolation was undertaken in the hope that the ribonuclease could either be washed from the preparation or denatured. The procedure cannot properly be said to follow that of any particular author, although any one step may have been a part of a previously published treatment. Frozen pancreas was ground into a cold 7 per cent solution of trichloroacetic acid. The pH was maintained just acid to methyl orange indicator. The suspension was blended with an electric mixer which was surrouned by a pack of dry ice. After filtration by suction with a layer of Super-Cel on the paper, the cake was resuspended into 1 liter of a cold 2 per cent solution of trichloroacetic acid and refiltered. This procedure was repeated twice. The filter cake was then washed five times with 1 liter portions of cold water. Finally the material was suspended and washed with 1 liter portions of the following alcoholic solutions: 15, 35, 35, 50, 70, and 95 per cent. Separations were accomplished with the aid of a Servall "hi-speed" centrifuge. After the separation, the material was dispersed in an ether-alcohol mixture (1:3), warmed on a steam bath, and filtered. The filter cake was ground into a chloroform-alcohol mixture (1:1) and refluxed for 30 minutes. After filtration the cake was washed with an ether-alcohol mixture (1:1) and dried in air. Qualitative tests for ribonuclease activity were positive. The nucleic acid which was isolated from the nucleoprotein had a total nitrogen to total phosphorus ratio of 1.94 and a ratio of labile phosphate to total phosphate of 71 per cent. These results are again similar to those for the first two preparations.

The first three preparations definitely carried ribonuclease into the final stage, in which action of the ribonuclease with the pentose nucleic acid could be assumed. Therefore a fourth preparation was undertaken. Pancreas which was obtained in the same manner as described for the second preparation was extracted to remove ribonuclease by the method of Kunitz (14). The filter cake was then extracted twice with a 50 per cent solution of acetone according to the procedure of Dubos and Thompson (9). The air-dried filter cake was then suspended in water, neutralized to pH 6.0, and the extraction of nucleoprotein undertaken by the technique of Jorpes. However, the extractions and alcoholic precipitation procedures yielded a nucleic acid slightly contaminated with protein rather than a nucleoprotein. Removal of the contaminating proteins by the method of Sevag, Lackman, and Smolens yielded the sodium salt of a nucleic acid with the following composition: total nitrogen 13.9 per cent, ratio of guanine nitrogen to total nitrogen 0.43, ratio of purine nitrogen to total nitrogen 0.67, total phosphorus 8.1 per cent, ratio of labile phosphate to total phosphate 48.5 per cent, ratio of total nitrogen to total phosphorus 1.72.

The non-dialyzable residues which remained after ribonuclease action on ribonucleic acid from yeast, as well as the non-dialyzable residues which remained after the action of ribonuclease on certain of the foregoing preparations of pentose nucleic acid from pancreas, were prepared by the methods described in previous publications (8, 13). The results of the analyses are presented in Table I.

Table I

Composition of Non-Dialyzable Residues (Sodium Salts) Obtained by Action of Ribonuclease on Ribonucleic Acid from Yeast and Pentose Nucleic Acid from Pancreas

	Ribonucleic acid		Pentose nucleic acid	
Total N, %	15.45	15.40	15.45	15.30
$\frac{\text{Guanine N}}{\text{Total N}}$, %	48	47	58	51
$\frac{\text{Purine N}}{\text{Total N}}$, %	81	82	78	82
Total P, %	8.08	8.14	8.20	7.88
$\frac{\text{Labile P}}{\text{Total P}}$, %	70	69	68	70
$\frac{\text{Total N}}{\text{Total P}}$	1.92	1.89	1.88	1.94

DISCUSSION

The compositions of the first three preparations of pentose nucleic acid from pancreas are similar to those previously reported in the literature in that the contents of purines and particularly of guanine are relatively high. Since it was demonstrated that ribonuclease was contained in the pancreatic nucleoprotein, which is the intermediate in the isolation of pentose nucleic acid from pancreas by classical procedures, it can be inferred that part of the non-dialyzable fragments are the result of action by trace amounts of ribonuclease upon the original nucleic acid. Thus, it is believed that all samples of pentose nucleic acid from pancreas, which have been isolated to date, represent degradation products of the native nucleic acid.

The fourth preparation of pancreatic nucleic acid is, to our knowledge, the first reported isolation attempted after the removal of ribonuclease. The method chosen for the extraction of ribonuclease, while considered a standard procedure for the isolation of ribonuclease, may bring about certain changes, such as hydrolysis of certain labile bonds, in the nucleic acid. This can only be determined by further study. However, with the exception of the slightly higher quantity of guanine, which is the lowest yet reported for such a preparation, the analyses are remarkably similar to those reported for certain preparations of ribonucleic acid from yeast.

The compositions of the non-dialyzable residues which were produced from both ribonucleic acid from yeast and the first two preparations of pentose nucleic acid from pancreas, after complete action by ribonuclease followed by exhaustive dialysis, are similar. Both types of residues are characterized by increased content of purines and particularly of guanine, in comparison to the composition of the original preparations of the nucleic acids. The manner of formation of the four residues, in addition to the similar compositions, suggests that the residues have structural similarities. These structural similarities must therefore exist in the native nucleic acids. Present information does not permit speculation regarding the fragments which are the result of ribonuclease action and which are removed by dialysis. They could be quite different for the two types of nucleic acids. Schmidt, Cubiles, and Thannhauser (15) have recently emphasized certain differences between the behavior of the two types of nucleic acids toward ribonuclease. Their findings can be construed according to the foregoing arguments or that the pancreas does contain a pentose nucleic acid which is resistant to the enzyme.

SUMMARY

It has been demonstrated that ribonuclease is contained in the pancreatic nucleoprotein which is the intermediate in the isolation of pentose nucleic acid from pancreas by classical procedure.

It is believed that all samples of pentose nucleic acid from pancreas reported to date represent degradation products of the native nucleic acid.

The analytical data are given for a preparation of pancreatic nucleic acid that has been isolated after removal of ribonuclease from the pancreas.

The non-dialyzable residues which are obtained by the action of ribonuclease on the pentose nucleic acid from pancreas are compared with those which are obtained in analogous experiments in which ribonucleic acid from yeast is the substrate.

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SOME PROPERTIES OF A FACTOR IN ALFALFA MEAL CAUSING DEPRESSION OF GROWTH IN CHICKS

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It has recently been demonstrated that dehydrated alfalfa meal in the diet of young chicks causes a depression in growth proportional to the level of alfalfa meal in the ration (1, 2). The growth depression is accompanied by a decline in food consumption. Most of the inhibitory effect on growth can be removed by exhaustive extraction of the alfalfa meal with hot water. The residue so produced has little growth-depressing action, whereas the water extract produces marked inhibition of growth (2).

The present report describes a crude fractionation of this water extract and some of its properties.

EXPERIMENTAL

Fractionation—The general procedure employed in the fractionation may be broadly outlined as follows: Dehydrated alfalfa meal was extracted overnight with a large volume of distilled water at room temperature. Toluene and chloroform were added to inhibit bacterial growth. The mixture was filtered and the residue extracted five times with a large volume of distilled water at 95°, the extract being pressed out each time in a filter press. The residue was dried at 80°. The combined filtrates were concentrated *in vacuo* and 95 per cent ethyl alcohol (sp. gr. 0.815) was added until a concentration of 80 per cent alcohol was reached. The resulting precipitate was allowed to settle for several hours and filtered; it was then suspended in boiling water, alcohol was once more added to a concentration of 80 per cent, and the mixture was again filtered. After the precipitate was washed with 80 per cent and 95 per cent alcohol, it was dried at 65°.

The combined alcohol filtrates were concentrated *in vacuo* to a volume of 1 liter per 4 kilos of alfalfa meal, and to this solution were added 2.66 volumes of 95 per cent ethanol. Acetone was then added with constant stirring. Upon first addition of acetone a dark brown syrupy mass separated out. Further addition of acetone precipitated a light brownish yellow substance which agglomerated into a gummy mass. Fractional reprecipitation of this mixture of acetone-insoluble precipitates yielded two fractions, Nos. I and II. Fraction I was a dark brown hygroscopic syrup which was extremely difficult to dry. Fraction II was obtained as a gummy yellowish mass which dried to a light brown solid. The supernatant liquids from the numerous reprecipitations of the acetone-insoluble material were decanted through filter paper and combined. After standing for several days, a white crystalline substance (Fraction III) precipitated from this solution.

When possible, the experimental fractions were dried and finely ground. Those that were difficult to dry were dissolved in 50 per cent ethanol and dried on the barley used in the experimental diet.

Growth Tests—The biological tests employed to detect the presence or absence of the growth-inhibiting factor were growth tests on 7 to 14 day-old single comb white Leghorn chicks that had been previously maintained on a standard stock diet. The chicks were selected for uniformity of weight and rate of growth and were maintained on the experimental diets for periods varying from 2 to 3 weeks. The basal diet, which was formulated to fulfil all the known nutritive requirements of the chick, consisted of the following ingredients: ground barley, 30 per cent; ground corn, 22 per cent; ground wheat, 10 per cent; wheat bran, 10 per cent; fishmeal, 7.5 per cent; soy bean oil meal, 10 per cent; dried whey, 2.5 per cent; dried skim milk, 2.5 per cent; liver meal, 2 per cent; ground limestone, 1.5 per cent; special steamed bone meal, 1.2 per cent; sodium chloride, 0.5 per cent; $MnSO_4 \cdot H_2O$, 0.025 per cent; fortified fish oil (containing 3000 units of vitamin A and 400 of vitamin D per gm.), 0.25 per cent; and riboflavin, 2 mg. per kilo. The stock diet was used as a control diet. It contained the same ingredients as the basal diet, except for the barley, which was replaced by the following constituents: corn, 8 per cent; wheat bran, 4.5 per cent; alfalfa meal, 4 per cent; barley, 10 per cent; and wheat, 3.5 per cent. The basal diet and the stock diet produced parallel growth results in all trials. The experimental diets contained either 20 per cent alfalfa meal or the prepared fractions. These were substituted for a like quantity of barley in the basal diet. The 20 per cent level of alfalfa meal had been found consistently to produce growth depression. The various fractions prepared from alfalfa meal were usually fed at levels somewhat above that which would be equivalent, on the basis of solids, to 20 per cent alfalfa meal, in order to allow for losses in preparation or for incomplete extraction.

The results recorded in Table I show that the growth depressant may be extracted by water and that repeated extraction with hot water is more efficient than extraction at room temperature.

Neither the precipitate obtained with 50 per cent alcohol nor that obtained with 80 per cent alcohol had growth-depressing action (Table II). The precipitate obtained by addition of acetone to an aqueous alcohol solu-

TABLE I

Growth-Depressing Effects of Aqueous Extracts of Alfalfa Meal

8 day-old chicks, eight chicks per group.

Diet	Equivalent of alfalfa	Average gain in weight, 21 days
	per cent	*gm.*
20% alfalfa	20	85
15% residue cold water extraction	21	129
13% " hot " extraction	20	151
13% " cold " and hot water extraction	21	156
7% cold water extract	34	79
7% hot " "	29	62
5.25% hot water extract of cold water extracted residue	70	52
Stock diet	4	169
Basal " (no alfalfa)	0	184

TABLE II

Effect on Growth of Chicks of Various Fractions Obtained from Aqueous Extract of Alfalfa Meal

Eight chicks per group.

	Diet	Equivalent of alfalfa	Average gain in weight	
			14 days	21 days
		per cent	*gm.*	*gm.*
Trial I, 9 day-old chicks	20% alfalfa	20	38	
	2.4% 50% ethanol ppt.	70	81	
	7% filtrate solids	37	35	
	Basal diet (no alfalfa)	0	81	
Trial II, 7 day-old chicks	20% alfalfa	20	32	57
	3% 50% ethanol ppt.	83	92	
	2.8% 80% ethanol ppt.	89	89	
	5% ethanol-acetone ppt.	90	21	37 (4 dead, 7 dead by 36th day)
	5% ethanol-acetone filtrate	55	69	130
	Stock diet	4	85	149
	Basal "	0	88	157

tion had marked growth-depressing activity, but the filtrate had almost none (Table II). Of eight birds on the diet containing the alcohol-acetone precipitate, four were dead within 3 weeks, and seven were dead by the 36th day. Those birds examined immediately after death were extremely emaciated, showing atrophy of the leg and breast muscles, ascites and watery blood; the general appearance resembled starvation, although the alimentary tract in some cases was found to contain food.

During the course of the fractionation it was noted that the aqueous solutions of the fractions later found to have growth-depressing properties foamed strongly. This characteristic suggested the presence of saponins, and thus a possible explanation of the toxicity of alfalfa meal to chicks. It was first shown by Ransom (3, 4) that saponins react with sterols to form addition compounds which no longer possess toxic properties and which have lost the ability to hemolyze red blood cells. To test the possibility that a sterol might counteract the growth-depressing agent, cholesterol was added to an alfalfa-containing diet.

The results of this test indicated that cholesterol largely counteracted the growth-depressing action of alfalfa meal (Table III). In further experiments, which need not be described here, cholesterol was found repeatedly to have a definite effect in counteracting the growth depressant.

TABLE III

Effect of Cholesterol on Toxicity of Alfalfa Meal

2 week-old chicks, twelve chicks per group.

Diet	Average gain in weight	
	14 days	21 days
	gm.	gm.
20% alfalfa	56	117
20% " + 0.5% cholesterol	90	161
20% " + 1% cholesterol	102	176
Basal diet (no alfalfa)	117	197

An experiment was then carried out to determine the relative potency of the inhibitory fractions (Table IV). Fractions I and II and the alfalfa meal were fed both with and without the addition of cholesterol. The acetone precipitate and the fractions obtained from it (Fractions I and II) were found to be more active as growth depressants than the original aqueous extract, indicating considerable concentration of the growth inhibitor. The toxicity of both Fractions I and II was to a large degree counteracted by cholesterol. The group fed Fraction I was maintained only 12 days because of lack of material. Fraction III was not tested in this trial since it had been found in a preliminary experiment to have no growth-depressing action. In one group (Table IV) a phytosterol (Eastman), of unknown origin, appeared also to have some effect in counteracting the growth inhibition.

Hemolytic Tests—If saponins were responsible for the toxicity of alfalfa meal, the growth-depressing fractions should possess hemolytic properties which should be counteracted by treatment with cholesterol. All fractions were made up in duplicate as 1 per cent solutions in 0.9 per cent sodium chloride. Digitonin, a saponin of known hemolytic activity, was used as a control, in 0.1 per cent solutions. To one of each pair of duplicate solu-

TABLE IV

Growth-Depressing Effects of Various Fractions from Alfalfa Meal

9 day-old chicks, eight chicks per group.

Diet	Equivalent of alfalfa	Average gain in weight	
		12 days	21 days
	per cent	*gm.*	*gm.*
20% alfalfa	20	28	72
20% " + 1% cholesterol	20	61	137
3% aqueous extract	10.7	67	153
3% ethanol-acetone ppt	31	45	94
3% Fraction I	133	36	
3% " " + 1% cholesterol	133	82	
3% " II	41	40	85
3% " " + 1% cholesterol	41	71	154
Basal diet (no alfalfa)	0	78	168
Stock "	4	79	174
20% alfalfa + 1% phytosterol (Eastman)	20	52	126

TABLE V

Comparison of Growth Depression and Hemolysis by Various Fractions from Alfalfa Extract

Sample	Growth depression*	Hemolysis†
Hot water extract	+	+
" " " + cholesterol	Not tested	−
80% alcohol ppt	−	−
Alcohol-acetone ppt	+	+
" " + cholesterol	Not tested	−
Fraction I	+	+
" " + cholesterol	−	−
" II	+	+
" " + cholesterol	−	−
" III	−	−

* + indicates growth depression, − little or no growth depression.

† + indicates hemolysis, − no hemolysis.

tions of alfalfa fractions and of digitonin was added enough cholesterol to make a 0.5 per cent suspension. All solutions were then heated for $\frac{1}{2}$ hour in a boiling water bath, allowed to stand for several hours, and filtered. The hemolytic properties of each solution were then determined, by the use of a 2 per cent suspension of washed rat erythrocytes in isotonic saline solution as in the procedure described by Kofler (5).

All samples having growth-depressing action also had hemolytic action. The cholesterol treatment prevented hemolysis in all instances (Table V). Samples that did not depress growth did not hemolyze erythrocytes. All samples except Fraction I caused complete hemolysis at a dilution of 1:800; Fraction I caused complete hemolysis at 1:400. Similar results were obtained by substituting one sample of chicken erythrocytes for rat erythrocytes. The hemolytic index was not determined, since none of the samples was considered sufficiently pure for this test.

DISCUSSION

Fractions obtained from an aqueous extract of alfalfa meal produced depression of growth in chicks. The strong foaming properties of these fractions suggested saponins as the growth-depressing agent. Experimental evidence for this possibility was obtained in the finding that growth-depressing fractions of alfalfa hemolyzed red cells and that treatment with cholesterol inactivated the hemolytic properties. Further evidence was found in feeding tests, which indicated that the growth-depressing activity of alfalfa could be largely counteracted by the inclusion of cholesterol in alfalfa-containing rations.

Reports in the literature concerning the presence of saponins in alfalfa are extremely few. Jacobson (6) in 1919 prepared from alfalfa a substance which foamed strongly in aqueous solutions and which he described as a saponin. Since this material contained nitrogen and since it failed to hemolyze red blood cells, some authors have doubted that it should be considered a saponin (5, 7). Jaretzky, however, using the hemolytic test, found evidence for the occurrence of saponins in twenty species of *Medicago*, including *Medicago sativa* (alfalfa) (7, 8). It is therefore possible that Jacobson's "alfalfa saponin" was a crude preparation and might have undergone some inactivation owing to the method of preparation.

Until saponins are isolated from alfalfa meal in pure form and are shown by feeding tests to cause growth depression, it cannot be stated categorically that they are responsible for the growth-inhibiting properties of alfalfa meal. The present report, however, strongly indicates that any further investigations of the deleterious component, or components, of alfalfa must consider the possibility that saponins may account wholly or in part for its growth-inhibiting effects.

SUMMARY

1. Concentrates which depressed the growth of chicks were obtained by fractionating a hot water extract of alfalfa meal. The active principle was soluble in 50 and 80 per cent ethanol and was largely precipitated from an aqueous alcohol solution by the addition of acetone.

2. The growth-depressing action of this material was to a great extent counteracted by simultaneous feeding with cholesterol.

3. The inhibitory concentrates had hemolytic properties which were destroyed by boiling the concentrates with cholesterol. The hemolytic properties, together with the observed foaming action, strongly suggest that the material involved is a saponin.

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THE BIOLOGICAL ACTIVITY OF α-TOCOPHERYLHYDROQUINONE AND α-TOCOPHERYLQUINONE*

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A major obstacle in relating the biochemical action of α-tocopherol with an oxidation-reduction system in the body has been the large and apparently convincing volume of evidence indicating that its *p*-quinone, α-tocopherylquinone, and α-tocopherylhydroquinone are devoid of vitamin E activity.

Thus Karrer and coworkers (2), John, Dietzel, and Emte (3), Wright and Drummond (4), Karrer and Geiger (5), and Golumbic and Mattill (6) found that the quinone was inactive in the rat. Golumbic and Mattill also reported that the hydroquinone, α-tocopherylhydroquinone, was inactive in this species. These same workers (7) found no activity when α-tocopherylquinone was administered orally or parenterally to rabbits deficient in vitamin E. While Evans *et al.* (8) reported the *p*-quinone to be almost as effective as α-tocopherol itself in the rat, a 4 mg. dose producing fertility in half of the animals tested, later experiments by Tishler and Evans (9) showed that 100 mg. doses of a well characterized preparation were devoid of activity.

Utilizing the methods employed by Mackenzie and McCollum (10) in their demonstration of the cure of nutritional, muscular dystrophy in rabbits by α-tocopherol, we have found that both α-tocopherylquinone and the hydroquinone are potent sources of vitamin E when injected intravenously. Furthermore, both of these compounds have been shown to cure effectively muscular dystrophy when administered in higher doses *per os*.

Materials and Methods

α-Tocopherylquinone was prepared by oxidizing 2 gm. of *dl*-α-tocopherol[1] with 10 gm. of ferric chloride in 200 ml. of absolute ethanol for 24 hours at

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* The authors wish to express their appreciation to the Armour Fund for Research in Muscular Disease and to the Nutrition Foundation, Inc., for funds that greatly aided in these experiments. A preliminary report on a part of this work was presented before a Conference on Vitamin E, New York, April 16, 1949 (1).

[1] We are indebted to Merck and Company, Rahway, New Jersey, for generous supplies of *dl*-α-tocopherol.

room temperature in the dark. At the end of this time the reaction mixture was diluted with water and extracted with ethyl ether. The ether was removed *in vacuo* under nitrogen and the residue was dissolved in petroleum ether. The quinone was converted to the hydroquinone by hydrogenation with 5 per cent palladium on calcium carbonate as the catalyst. The catalyst was removed by centrifugation and the petroleum ether solution was then chilled at 0°, according to the procedure of Tishler and Wendler (11), whereupon the hydroquinone separated as a waxy solid. The mixture was centrifuged and the solvent was decanted. The α-tocopherylhydroquinone was washed several times with petroleum ether to remove any α-tocopherol or quinone present as a contaminant.

The hydroquinone was dissolved in ethyl ether and reconverted to the quinone by oxidation with silver oxide. The quinone so obtained was reduced and reoxidized by the procedures outlined above until its extinction coefficient did not increase. The extinction coefficients of the two preparations of quinone used in the biological tests were 450 and 455 at 270 mμ. The per cent of reducing substances present in these preparations, expressed as α-tocopherol, was 0.06 and 0.2, respectively, as measured by the method of Emmerie and Engel (12). The hydroquinone prepared by reducing these quinone preparations showed selective absorption at 288 mμ with an extinction coefficient of 88. The purity of both the quinone and hydroquinone was confirmed by infra-red absorption.[2]

The α-tocopherylhydroquinone employed in the animal tests was prepared immediately before its administration by hydrogenation of the purified quinone suspended in propylene glycol. The catalyst was removed from the hydroquinone, which is soluble in propylene glycol, by centrifugation.

The experimental animals were young male and female chinchilla rabbits weighing from 500 to 700 gm. Muscular dystrophy was produced by maintaining the animals on Diet 13 of Goettsch and Pappenheimer (13). Supplements were administered when the rabbits had developed Stage II dystrophy as defined by Mackenzie and McCollum (10). This stage of dystrophy is characterized by loss of weight, decreased food consumption, a creatine excretion of approximately 80 mg. daily, and moderate paralysis. Food was removed for 5 hours before and 5 hours after the administration of oral supplements to minimize mixing of the test substance and the experimental diet in the stomach. Injected animals were allowed continuous access to the diet. Oral doses were given by stomach tube and injected doses were given in the marginal ear veins.

For purposes of supplementation, the hydroquinone was dissolved in propylene glycol, or in propylene glycol containing 10 per cent ethanol.[3] A concentration of 20 mg. per ml. was generally employed. When amounts of the hydroquinone larger than 20 mg. were administered, the concentration was increased so that the volume did not exceed 1 ml. Immediately after the administration of the test dose, the hydroquinone content of each preparation employed was determined in an aliquot by a modification of the method of Emmerie and Engel. α-Tocopherylquinone and α-tocopherol were suspended in a minimal quantity of the propylene glycol-ethanol mixture (less than 0.5 ml.) immediately before administration.

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[2] Rosenkrantz, H., unpublished data.

RESULTS AND DISCUSSION

The results of our first experiments on the intravenous administration of single doses of α-tocopherylhydroquinone and α-tocopherylquinone to rabbits with Stage II muscular dystrophy revealed that both of these compounds possess vitamin E activity. The creatine excretion was depressed, muscular weakness disappeared, growth was resumed, and appetite returned to normal. The prompt drop in creatine excretion and the rate of fall are illustrated in Fig. 1.

In view of these positive results, the vitamin E activity of α-tocopherylhydroquinone, α-tocopherylquinone, and α-tocopherol was compared by the assay procedure described earlier by Mackenzie (14). This method is based on the correlation between the amount of α-tocopherol administered in a single dose and the duration of the fall in urinary creatine. The results of our studies on the comparative activity of these compounds administered intravenously are summarized in Table I. At a 5 mg. level the activity of the hydroquinone was approximately equal to that of α-tocopherol. However, increasing the amount of hydroquinone injected over a range of 5 to 25 mg. did not prolong the period of depressed creatinuria, indicating that excess amounts were not stored but were either excreted or destroyed. With α-tocopherol, on the other hand, increasing the dose prolonged the response. This finding demonstrated that the activity of the hydroquinone was not due to the presence of α-tocopherol as a contaminant.

Intravenous α-tocopherylquinone was less active than the hydroquinone (Table I). At the 5 mg. level the quinone had no effect on the creatinuria. While a definite response was produced by 10 mg., it was not until the dose was raised to 50 mg. that the duration of the effect was comparable to that obtained with 5 mg. of the hydroquinone. It should be noted, however, that the injected hydroquinone was in solution, whereas the quinone was suspended in the menstruum.

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[3] A suspension of the hydroquinone in 0.9 per cent NaCl, while not satisfactory for routine use because of its physical properties, produced the same physiological response. Propylene glycol itself has no curative action in dystrophic rabbits.

Vitamin E activity was also observed when single doses of the hydroquinone and the quinone were administered by stomach tube. As shown in Table II, the hydroquinone was the more active of the two compounds. In both cases, however, there was greater activity when the compounds were injected intravenously than orally. Presumably, the reduction in activity of both substances was due to poor absorption, to partial destruction in the gastrointestinal tract, or to inactivation in the body before they reached the systemic circulation. In any event, the lower potency of the quinone and hydroquinone when administered *per os*, and the inability of the body to store them efficiently, may explain the failure of other workers to detect their vitamin E activity.

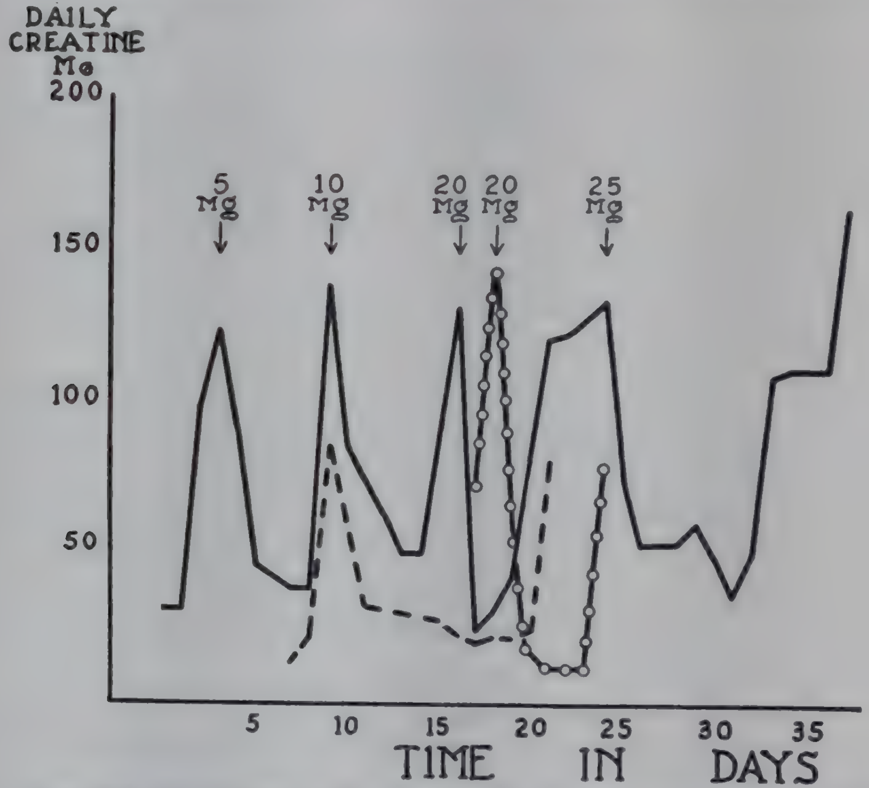

FIG. 1. Depression of creatinuria in dystrophic rabbits following the intravenous administration of single doses of α-tocopherylhydroquinone, α-tocopherylquinone, and α-tocopherol. Solid curve, Rabbit 17, α-tocopherylhydroquinone; broken line, Rabbit 27, α-tocopherol; ○, Rabbit 90, α-tocopherylquinone.

Having observed that both the quinone and hydroquinone, when given in a single dose, elicited a response in the dystrophic rabbit qualitatively indistinguishable from that produced by α-tocopherol, we carried out ex-

TABLE I

Effect of Intravenous Administration of α-Tocopherylhydroquinone, α-Tocopherylquinone, and α-Tocopherol on Creatine Excretion of Dystrophic Rabbits

No. of animals	Supplement	Dose	Average daily creatine excretion at time of supplement	Average maximal drop in creatine excretion	Duration of lowered creatine excretion	
					Average	Extremes
		mg.	*mg.*	*per cent**	*days*	*days*
4	α-Tocopheryl-hydroquinone	5	120	71	5.5	4–6
2		10	115	63	7.3	6–7
1		15	102	71	8.0	
4		20	150	84	5.2	4–6
2		25	83	72	6.5	5–8
1		50	104	85	8.0	
2	α-Tocopherylquinone	5	113	0	0	
2		10	98	77	2.0	
6		20	120	85	3.0	2–5
4		50	117	72	4.8	4–6
6		100	145	89	12.6	8–16
2	α-Tocopherol	5	110	82	6.5	5–8
1		10	87	75	12.0	
2		20	112	82	14.0	13–15
1		50	113	93	26.0†	

* $\frac{\text{Maximal drop in creatine (mg.)}}{\text{Creatine (mg.) at time of supplement}} \times 100.$

† Animal sacrificed; creatine excretion 15 mg. at time of death.

TABLE II

Effect of Oral Administration of α-Tocopherylhydroquinone, α-Tocopherylquinone, and α-Tocopherol on Creatine Excretion of Dystrophic Rabbits

No. of animals	Supplement	Dose	Average daily creatine excretion at time of supplement	Average maximal drop in creatine excretion	Duration of lowered creatine excretion	
					Average	Extremes
		mg.	*mg.*	*per cent**	*days*	*days*
1	α-Tocopherylhydro-quinone	5	110	0	0	0
2		20	121	70	3.5	3–4
2		100	98	75	7.5	7–8
4	α-Tocopherylquinone	50	156	0	0	
2		100	122	73	4.5	4–5
2		200	104	70	7.5	7–8
3	α-Tocopherol	20	104	76	15.6	11–20

* $\frac{\text{Maximal drop in creatine (mg.)}}{\text{Creatine (mg.) at time of supplement}} \times 100.$

periments to determine whether or not they could replace α-tocopherol for longer periods of time. Three rabbits with Stage II dystrophy and with a creatine output of 130, 152, and 162 mg., respectively, were injected daily with 12 mg. of α-tocopherylhydroquinone.[4] In each of the animals the creatine excretion fell to within the normal range of 10 to 15 mg. per kilo of body weight within 2 days, and there was a prompt remission of physical symptoms. Two of the animals were sacrificed after 10 days and the third one at the end of 7 weeks. Histological sections were prepared from the thigh muscles of each rabbit and examined for the presence of the lesions of muscular dystrophy described by Goettsch and Pappenheimer (13). None were found.

Three control rabbits with creatine excretions of approximately 100 mg. per day had extensive muscle lesions. Moreover, Mackenzie, Levine, and McCollum (15) have shown that on the experimental diet employed muscle lesions invariably are present even in those rabbits exhibiting a mild creatinuria. It may be assumed, therefore, that lesions were present in our animals when therapy was initiated and that these lesions were cured and their recurrence prevented by the hydroquinone.

The effectiveness of the quinone in substituting for α-tocopherol as a source of vitamin E over prolonged periods was demonstrated by curing animals of repeated attacks of dystrophy with single doses of this compound administered either intravenously or orally. The injected doses ranged from 20 to 100 mg.; the oral doses were either 100 or 200 mg. Five rabbits were cured of from three to seven attacks of Stage II dystrophy during periods that ranged from 11 to 19 weeks. Had they been given no supplement, these animals would have succumbed to their first attack of the disease within a week or 10 days (10).

With the demonstration of the biological activity of α-tocopherylquinone and α-tocopherylhydroquinone, a major obstacle in relating the biochemical action of vitamin E with an oxidation-reduction system has been removed, and a reconsideration of its reactions in the body is necessary.

Michaelis and Wollman (16) have recently observed the reversible formation of the semiquinone of α-tocopherol *in vitro* and have suggested that the activity of vitamin E *in vivo* may be due to this reaction. It is of course possible that the vitamin E activity of the quinone and hydroquinone is due to the conversion of these substances to α-tocopherol in the body. However, in view of our results it is also possible that α-tocopherol is converted to the hydroquinone either directly, or by way of the

[4] To eliminate the trauma produced by the daily injection of 10 per cent ethanol in propylene glycol, a suspension of the hydroquinone in a 10 per cent aqueous solution of propylene glycol was used in this experiment.

quinone, or its biologically potent isomer which recently has been isolated by Boyer (17), and that the hydroquinone and its semiquinone form a biologically active oxidation-reduction pair.

Finally, and more probably, all of the compounds discussed above may be active in the body. Not one, but several oxidation-reduction pairs may exist, each of which fulfils a biochemical function. The elucidation of this problem depends in a large measure on the development of sensitive and specific methods for the determination of α-tocopherol and of its quinone and hydroquinone in biological materials.

SUMMARY

α-Tocopherylhydroquinone and α-tocopherylquinone prevent and cure the creatinuria, paralysis, and loss of weight caused by vitamin E deficiency in the rabbit. It may be concluded, therefore, that these compounds possess vitamin E activity.

In bioassays based on the duration of the drop in creatinuria in dystrophic rabbits, the hydroquinone was found to be more active than the quinone. Both compounds were more active when injected intravenously than when given by stomach tube.

At the 5 mg. level the hydroquinone administered intravenously was approximately as active as α-tocopherol. However, in contrast to the results obtained with α-tocopherol, increasing the amount injected did not prolong the duration of the response. Apparently the rabbit's ability to store the hydroquinone is limited, and excess amounts are either destroyed or excreted.

The implications of these results with special reference to the participation of vitamin E in an oxidation-reduction system in the body are discussed.

It is a pleasure to express our appreciation to Dr. Cosmo G. Mackenzie for his advice and help during the course of this study. The authors wish to acknowledge the technical assistance of Mrs. Paula V. Schroeder.

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METHEMALBUMIN

INTERACTION BETWEEN HUMAN SERUM ALBUMIN AND FERRIPROTOPORPHYRIN IX*

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Methemalbumin is an abnormal component of blood plasma in certain diseases associated with excessive hemolysis (1). It was first identified by Fairley (2) as a complex of serum albumin and ferriprotoporphyrin. The nature of the protein-heme binding in this pigment has received little study, though in the reduced form of the complex Keilin (3) has postulated linkage with the porphyrin and not with the iron. Methemalbumin is not known to function in catalysis or in oxygen transport, as do certain other heme proteins, and no physiological rôle has been assigned to it. Formation of the complex removes free hematin from the plasma and provides a mechanism for its transport in the blood stream.

The studies reported here were made to characterize further the interaction between crystallized human serum albumin and ferriprotoporphyrin, leading to the formation of methemalbumin. This system is of interest as an association reaction between metalloporphyrin and a coordinating substance such as has been studied by Clark, Taylor, Davies, and Vestling (4) and by Shack and Clark (5).

Materials and Methods

The *crystallized human serum albumin* used in these studies was kindly supplied by Dr. W. L. Hughes, Jr. Three different preparations were

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* At The Johns Hopkins University, this work was supported by a grant-in-aid to one of us (M. R.) from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council. At the Department of Physical Chemistry, Harvard Medical School, this work was originally supported by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institutes of Health. This paper is No. 84 in the series "Studies on the plasma proteins" from the Harvard Medical School, Boston, on the products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. A preliminary report of this work has been presented in *Federation Proc.*, **8**, 329 (1949).

used: albumin crystallized with the aid of chloroform (No. 179, recrystallized five times); albumin crystallized with aid of decanol (decanol 10, recrystallized twice (6)); and albumin crystallized as the mercury salt (No. 35 Hg, recrystallized five times (7)). Except when noted, the protein was dissolved in phosphate buffer of ionic strength 0.3 at pH 7.3. Albumin concentrations were based on micro-Kjeldahl analyses with a nitrogen factor of 6.25.

Ferriprotoporphyrin—Commercial crystallized hemin (ferriprotoporphyrin IX chloride) was purified by three further crystallizations from pyridine and chloroform according to the procedure of Hans Fischer (8). On standing in alkaline solution, the hematin was transformed into a compound which, although having an absorption spectrum similar to that of hematin, did not interact with albumin. It was therefore necessary to examine the conditions of stability of ferriprotoporphyrin with respect to this alkaline conversion product. Ferriprotoporphyrin could be separated from the non-binding compound by differences in solubility in the two-phase system: *n*-butyl alcohol and 0.1 M buffer, either borate or bicarbonate, pH 9.6. The ferriprotoporphyrin was distributed preferentially in the organic phase, whereas the impurity remained in the aqueous phase. After several washings of the organic phase with fresh buffer, pH 9.6, the purified ferriprotoporphyrin was returned to aqueous solution by adding an equal volume of chloroform to the butyl alcohol layer and extracting with 0.1 N (or 0.01 N) sodium hydroxide.

The results of a Craig type counter-current distribution (9) experiment are illustrated in Fig. 1. The ratio of the partition coefficients of the two solutes was so great that essentially complete separation was achieved in an eight plate transfer. The concentration of hematin initially dissolved in the buffer was kept at 250 mg. per liter or less. The partition coefficients varied with the concentration, and less complete separation was achieved at higher concentrations.

With this technique, it was found that 25 to 30 per cent of the inactive product was formed in a solution of ferriprotoporphyrin in 0.1 M borate buffer at pH 9.6 during a 3 hour period. In 0.1 N sodium hydroxide for 1 hour, 15 per cent conversion product was formed, while in 0.01 N sodium hydroxide only 7 per cent impurity was formed in the same time. For these studies, hemin was quickly dissolved in 0.1 N sodium hydroxide and was immediately diluted to a sodium hydroxide concentration of 0.001 N. Solutions of hematin containing no more than 5 per cent of the conversion product were prepared in this manner. The impurity did not measurably increase during several hours.

Titrations were carried out by diluting 1 part of the protein solution with 2 parts of the 0.001 N sodium hydroxide solution of hematin, resulting in an ionic strength of 0.1 and pH 7.4. The solutions were maintained at 38° for ½ hour to assure equilibrium, after which they appeared to remain stable for 24 hours or more. This stabilizing effect of protein was observed even in the presence of a large molar excess of ferriprotoporphyrin over that which could be bound by the protein; *i.e.*, 10 moles of ferriprotoporphyrin per mole of albumin. Hematin was diluted in this way with buffer

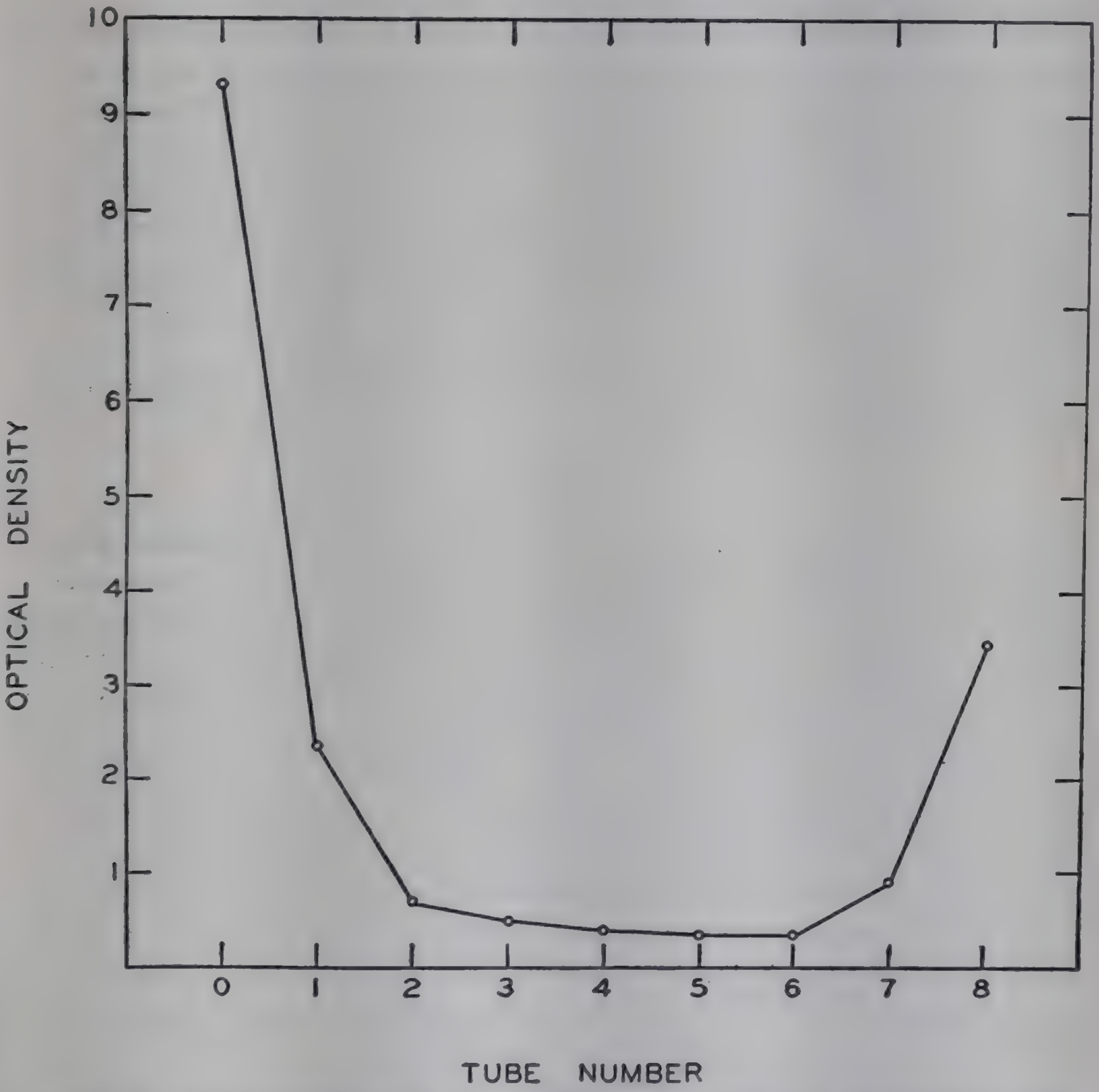

Fig. 1. Counter-current distribution pattern of a mixture of ferriprotoporphyrin and non-binding conversion product. Two phase solvent system; *n*-butyl alcohol and 0.1 M borate buffer, pH 9.6, in equal volumes. Tube 0 contained initially 250 mg. per liter of hemin which had stood 3 hours in the borate buffer. Optical densities at 403 mμ, 1 cm. path.

in the absence of protein as a blank. Readings were made immediately because ferriprotoporphyrin is unstable at pH 7.4 as a result of association and formation of aggregates. Optical density readings were not, as a rule, carried out at protein concentrations less than 0.1 mole per mole of ferriporphyrin, owing to the instability of the ferriporphyrin.

Titrations were carried out either in a series of tubes containing varying amounts of reagents read separately in the spectrophotometer, or by the use of a Gilmont type of ultramicro burette.[1] In the latter case, successive amounts of hematin solution were titrated directly into the buffered (pH 7.4, $\mu = 0.1$) albumin solution in a thermostat-controlled (38°) spectrophotometer cell, and the optical density was read a few minutes after each addition. This method had the advantage of requiring very small amounts of protein for a complete titration curve. It had the disadvantage that the hematin could have varied slightly with time.

Spectrophotometric measurements were carried out in a Beckman model DU spectrophotometer.

Results

Evidence of interaction with crystallized human serum albumin was obtained from the absorption spectra of ferriprotoporphyrin in the presence and absence of protein. These are shown in Fig. 2. In the presence of an equimolar amount of albumin, the broad absorption maximum of free ferriprotoporphyrin at 380 to 390 mμ (ϵ (385 mμ) $= 4.8 \times 10^4$) was replaced by the narrow band of methemalbumin with maximum absorption at 403 mμ (ϵ (403 mμ) $= 8.3 \times 10^4$). The protein-ferriporphyrin complex had absorption maxima at 500, 540, and 623 mμ. Ferriprotoporphyrin showed no discrete bands in the visible region between 400 and 650 mμ at pH 7.4, although in 0.1 N sodium hydroxide solution a band appeared at 610 mμ.

Dialysis experiments, which have proved useful in many studies of this kind, yielded only incomplete information, since it has been shown (5) from ultracentrifugal and diffusion measurements that ferriprotoporphyrin associates in aqueous solution to form aggregates of particle weight between 20,000 and 60,000. In an alkaline reaction in which association was diminished, conversion to the inactive form occurred. Since lengthy dialysis was necessary, these difficulties made it impossible to achieve equilibrium with respect to the ferriporphyrin. Experiments indicated, however, that the rate of diffusion of iron porphyrin from methemalbumin to protein-free buffer was very much slower than the rate of diffusion of free ferriprotoporphyrin to albumin.

Spectrophotometric Titrations—The most accurate and direct method of obtaining quantitative data appeared to be by direct titration of one re-

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[1] We have used the 0.1 ml. Gilmont ultramicro burette manufactured by the Emil Greiner Company.

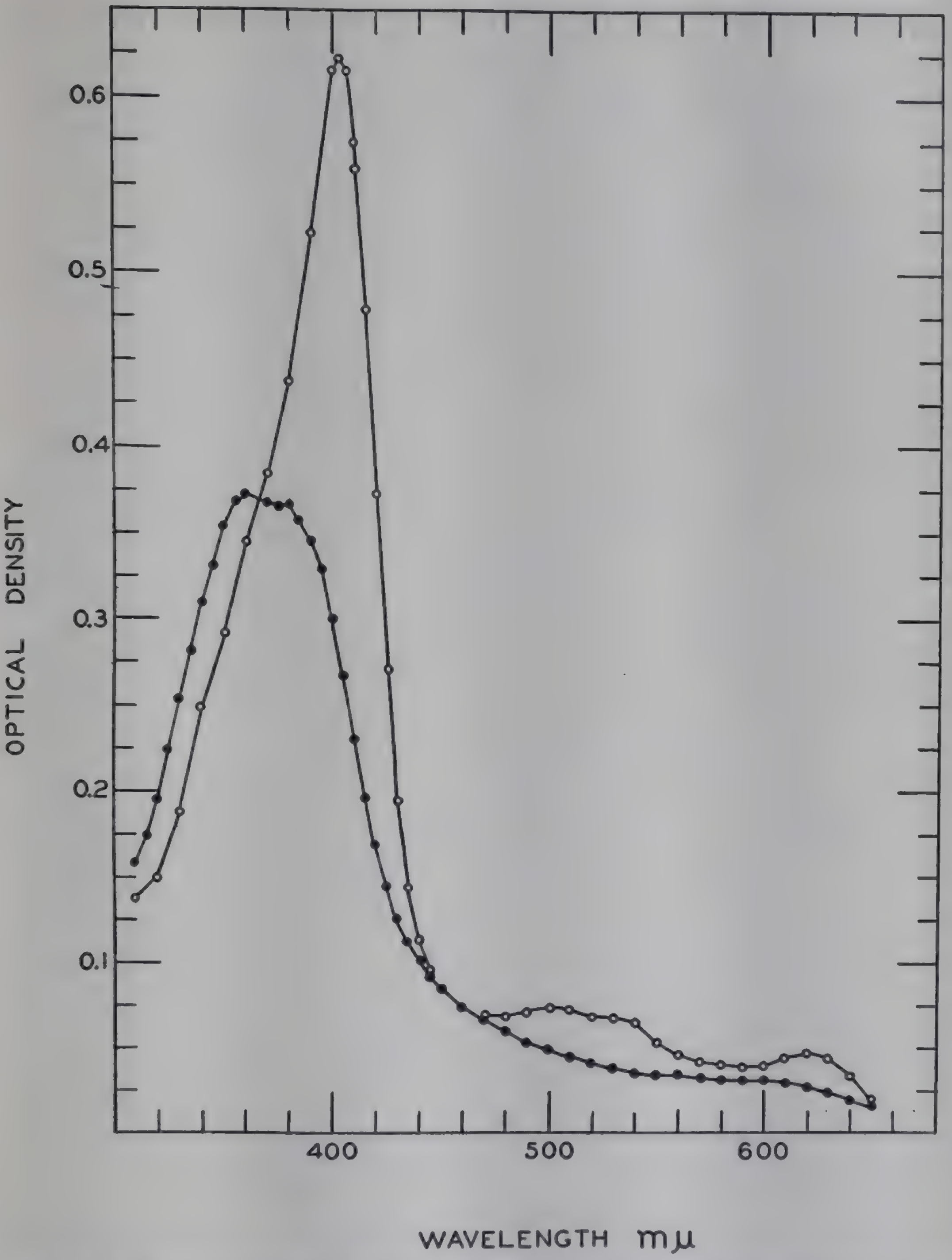

FIG. 2. Spectrophotometric curves of methemalbumin and ferriprotoporphyrin. ○, methemalbumin formed by combining ferriprotoporphyrin (5 mg. per liter as hemin, 7.7 × 10^{-6} M) and crystalline human serum albumin (0.53 gm. per liter, 7.7 × 10^{-6} M). ●, ferriprotoporphyrin, 5 mg. per liter as hemin. pH 7.4 phosphate buffer of ionic strength 0.1. Optical path, 1 cm.

actant with the other. To assure reproducibility, some measurements were made on the rate of reaction of ferriprotoporphyrin with albumin. Solutions of protein and ferriprotoporphyrin, at the desired temperature, were rapidly mixed in amounts to give a final albumin concentration of 2.1 gm. per liter and a hemin concentration of 10 mg. per liter in a 0.1 N borate buffer at pH 8.2. The molar ratio of albumin to ferriprotoporphyrin was 2:1. The reaction mixture was immediately transferred to the spectrophotometer cell in a cuvette chamber at constant temperature, and the rate of increase in extinction at 403 mμ was measured. The reaction took place rapidly and was 91 per cent complete in 30 minutes at 22° and complete in the same time at 38°. Solutions for measurement were therefore maintained at 38° for 30 minutes. Since the complex formed at 38° was stable at 22°, optical determinations were carried out at room temperature.

The data obtained on titrating a solution of albumin with ferriprotoporphyrin are presented in Fig. 3. The titration curve at a wave-length of 403 mμ consisted of an initial linear section, an intermediate zone of curvature, and a second linear section. The slope of this latter section, which was constant up to iron porphyrin concentrations in 40-fold molar excess over albumin, appeared to be chiefly due to the appearance of free ferriprotoporphyrin in the solution, and, to a lesser extent, to a non-specific enhancement of the extinction of free ferriporphyrin by protein. This is shown by the lower two curves in Fig. 3. The titration curve of γ-globulin with ferriprotoporphyrin consisted of a straight line whose slope was nearly the same as the second linear portion of the titration curve of albumin, and slightly greater than the slope for free ferriprotoporphyrin alone. A possible explanation of this non-specific effect is that both γ-globulin and albumin bound ferriprotoporphyrin to yield a product characterized by a low binding constant, or that the protein produced a change in the state of physical dispersion of iron porphyrin aggregates. In the case of γ-globulin, this appeared to be the only reaction; in the case of albumin it appeared to be secondary, and of minor extent in comparison to the primary interactions which were characterized by high binding constants and by a pronounced change in spectral absorption. Subtraction of the ferriprotoporphyrin reference line, corrected for the non-specific effect of protein, from the albumin titration curve (Fig. 4, upper graph) almost completely eliminated the positive slope of the second linear section of the curve.

The addition of ferriprotoporphyrin to albumin resulted in a linear increase in the optical density at 403 mμ (Figs. 3 and 4) until 1 mole of iron porphyrin per mole of albumin had been added. There followed a zone of curvature which extended to the addition of 2 moles of iron porphyrin. Beyond this end-point there was an accumulation of free ferriprotoporphyrin in solution. The over-all reaction can be represented as follows:

$$L + 2M \rightarrow LMM \tag{1}$$

The molecular extinction coefficient of the complex LMM (2 moles of ferriprotoporphyrin per mole of albumin), as determined from these data, was taken as $\epsilon_{LMM} = 15.0 \times 10^4$ (403 mμ).

When the spectrophotometric titration of albumin with ferriprotoporphyrin was measured at 623 mμ, the data indicated the binding of 4 moles of iron porphyrin per mole of albumin. Since this was not reflected in measurements at 403 mμ, the iron porphyrin bound after the first 2 moles appeared not to affect the Soret band (403 mμ) sufficiently to reveal the addition. The additional 2 molecules of ferriporphyrin may have interacted with the protein by a different mechanism from that which governed

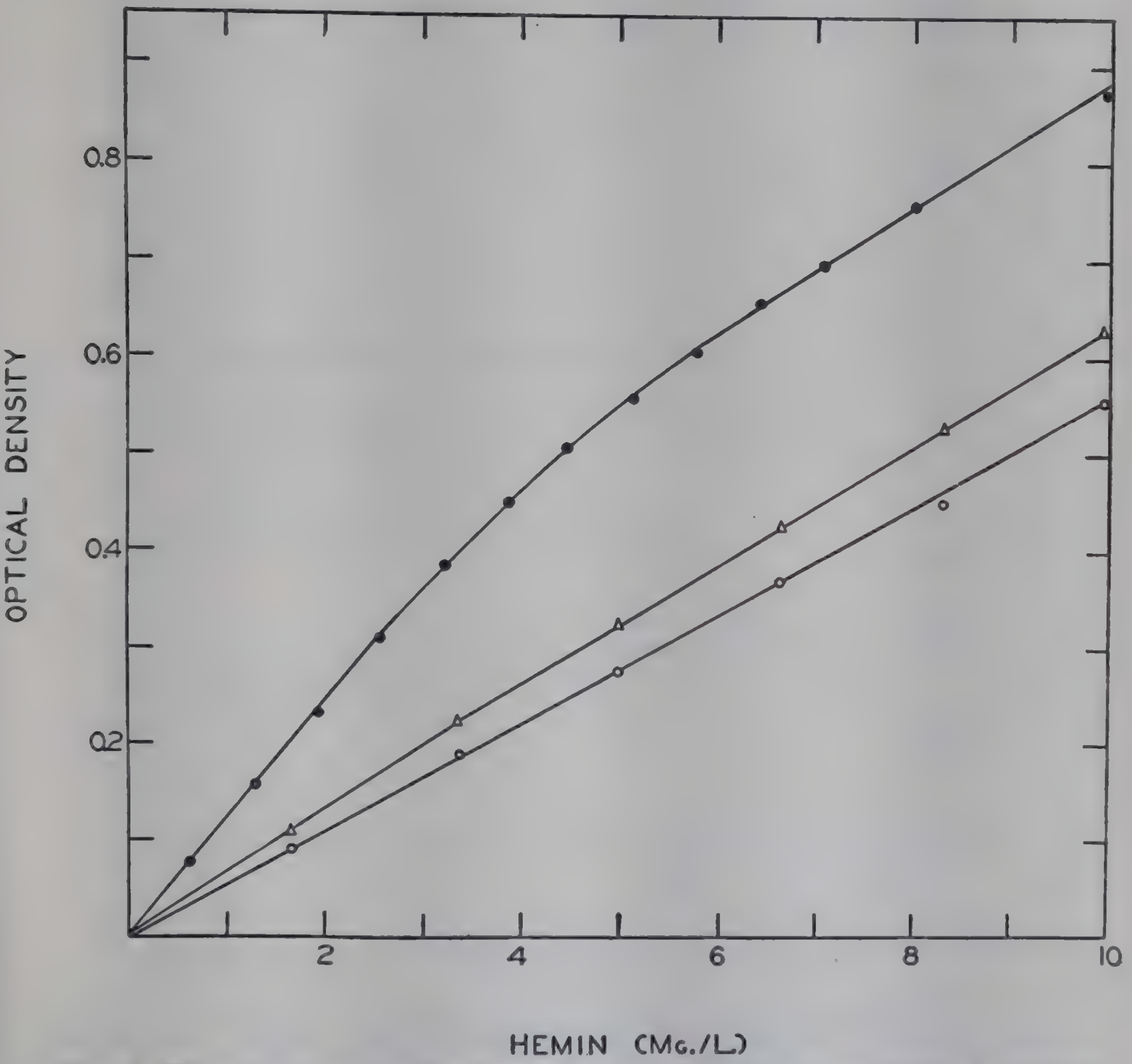

FIG. 3. Titration of crystalline human serum albumin and γ-globulin with ferriprotoporphyrin. ●, titration of serum albumin (0.266 gm. per liter, 3.85×10^{-6} M). The solid line was computed from equations. The curve has been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity. Δ, titration of γ-globulin, fraction II-1,2, 0.266 gm. per liter. ○, ferriprotoporphyrin reference line. Wave-length 403 mμ, 1 cm. path.

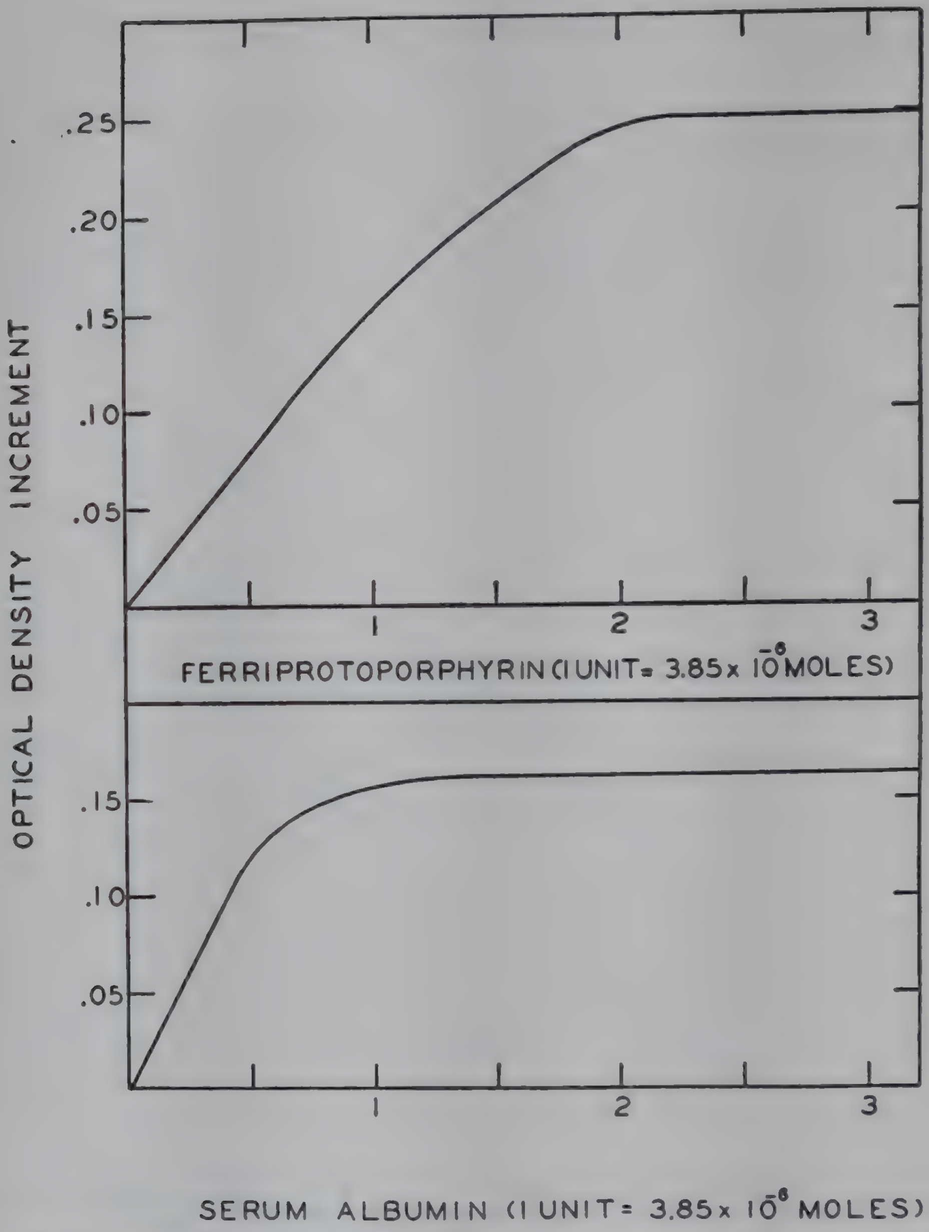

Fig. 4. Corrected titration curves. The curves represent the increment in optical density due to formation of methemalbumin (*LM* and *LMM*). The titration data have been corrected for absorption due to protein, ferriprotoporphyrin, and to the non-specific effect of protein. The curves are for ferriporphyrin of 100 per cent purity. Upper graph, titration of serum albumin (3.85×10^{-6} mole per liter) with ferriprotoporphyrin. Lower graph, titration of ferriprotoporphyrin (3.85×10^{-6} mole per liter) with serum albumin. Wave-length 403 mμ, 1 cm. path.

the binding of the first 2 molecules. This, however, would be an *ad hoc* assumption. In dealing with solutions of ferriporphyrin there is always the uncertainty that changes in state of aggregation may alter the absorption. Thus, what we have called the non-specific effect of γ-globulin was perhaps due, in part, to change in degree of dispersion of ferriporphyrin by this protein. In the absence of conclusive evidence we have tentatively treated the data on the simplest theoretical basis.

The converse type of titration in which serum albumin was added stepwise to a fixed amount of ferriprotoporphyrin is illustrated in Fig. 5. The end-point occurred when 1 mole of albumin had been added per mole of ferriprotoporphyrin (Fig. 4, lower graph, and Fig. 5). Accordingly, in the presence of excess albumin, the reaction was

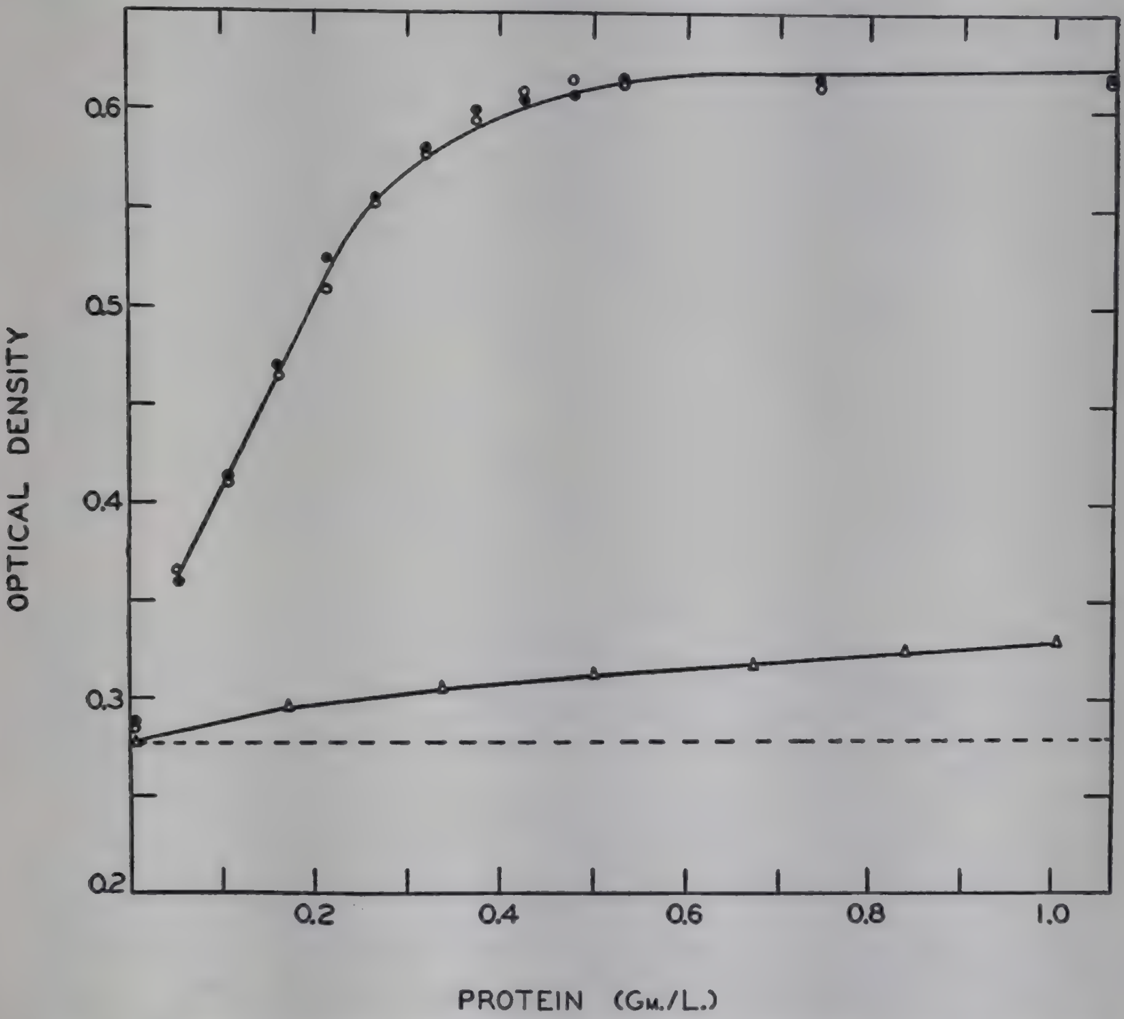

FIG. 5. Titration of ferriprotoporphyrin with crystalline serum albumin and with γ-globulin. Concentration of ferriporphyrin, 5 mg. per liter as hemin (7.7×10^{-6} M). ○ and ●, titration with serum albumin. Solid line computed from equations. The curve has been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity. Δ, titration with γ-globulin, fraction II-1,2. Wave-length 403 mμ, 1 cm. path.

$$L + M \rightarrow LM \tag{2}$$

Beyond the end-point, further increase in optical density could not be detected provided a slight increment was corrected by subtraction of a small protein blank. From these titration curves, ϵ_{LM} (403 mμ) has been taken as 8.3×10^4.

Influence of pH, Buffer Ions, and Ionic Strength—Methemalbumin (*LM*) was first formed by mixing ferriprotoporphyrin with albumin in an unbuffered 0.15 M sodium chloride solution. Buffer was then added, and the solutions were incubated another 15 minutes to permit reequilibration. The results of such an experiment are shown in Fig. 6. The two buffers, acetate and phthalate, were adjusted to give an ionic strength of 0.15 throughout the pH range. Isoelectric precipitation and denaturation of protein invalidated readings between pH 3.0 and 4.6. There was progressive increase in optical density from pH 4.6 to 7.0. In this pH range, density readings were higher in acetate than in phthalate, suggesting a specific ion effect. However, the two curves converged at pH 7.0 and remained constant between pH 7.0 and 10.0.

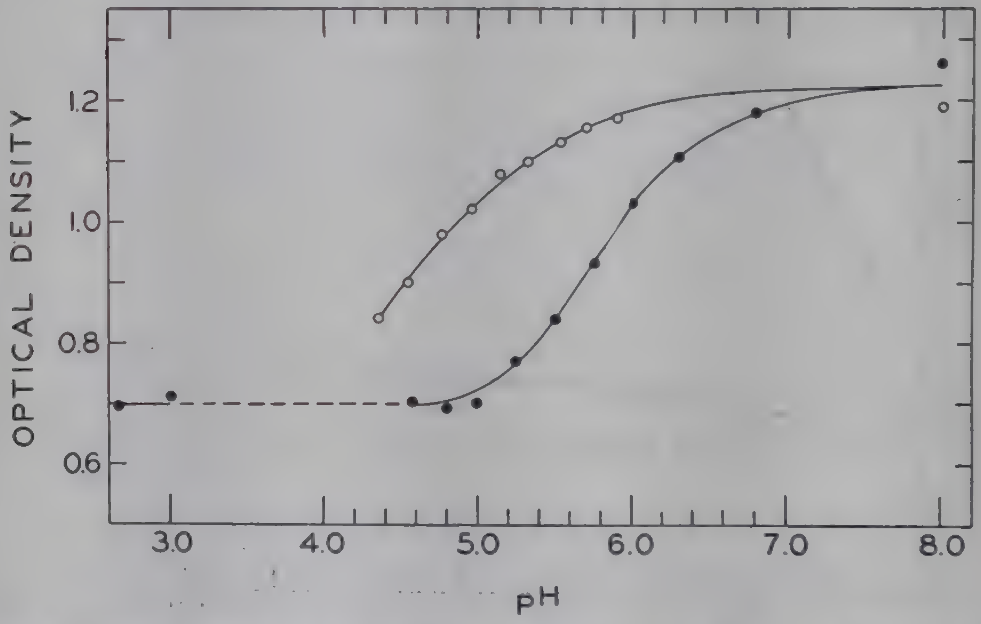

FIG. 6. Optical density of methemalbumin in relation to pH. ○, acetate buffer; ●, phthalate buffer. Ionic strength 0.15.

In another type of experiment, Fig. 7, ferriprotoporphyrin was titrated with protein in buffers of different composition and pH. Identical curves were obtained in phosphate, borate, and glycine buffers at pH 7.5, 9.0, and 9.9 respectively. Maximal optical density values, corresponding to complete binding, were reached when an equimolar amount of albumin was added to the ferriporphyrin. At more acid reactions, namely at pH 5.0 and 6.0 in acetate and phosphate buffers, the complex dissociated and complete binding of the ferriporphyrin was not attained until protein was

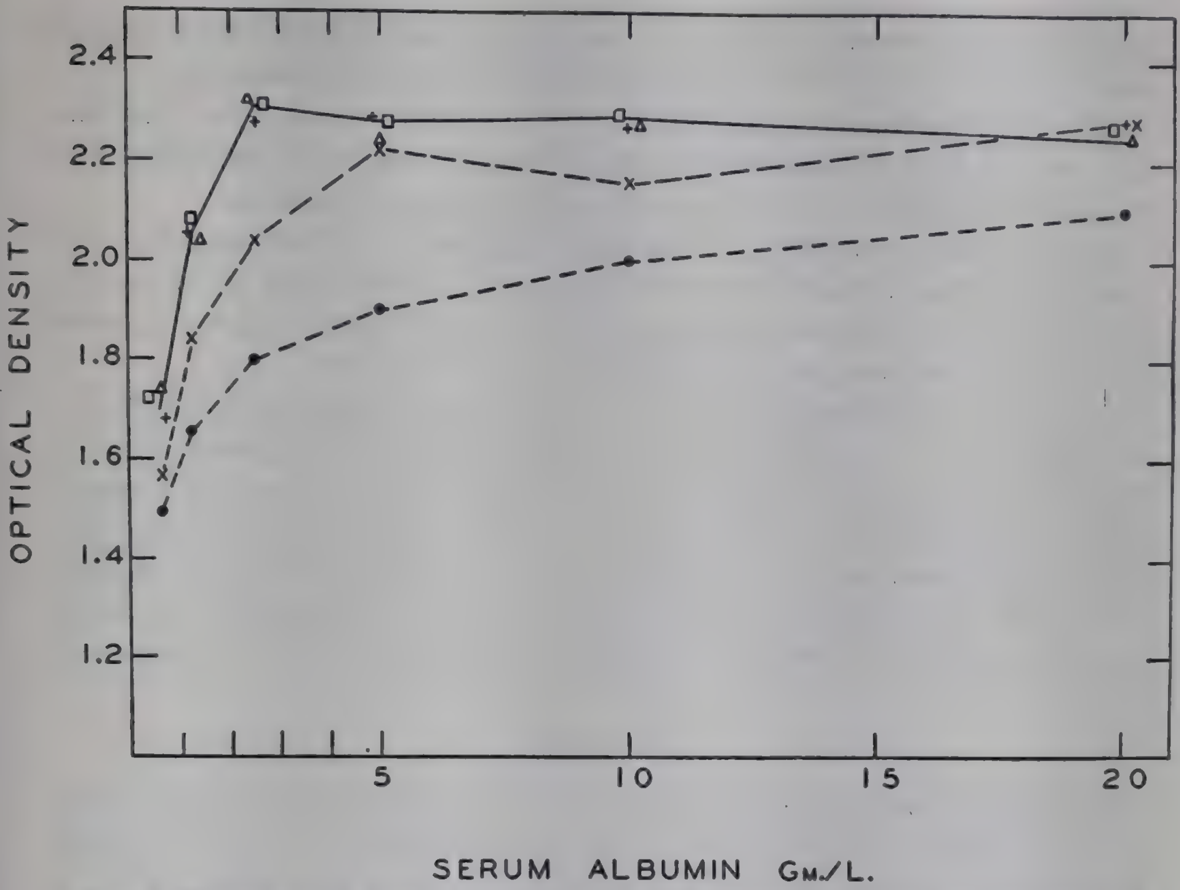

FIG. 7. Titration of ferriprotoporphyrin with serum albumin at various pH levels. Ferriporphyrin concentration, 20 mg. per liter as hemin (30.7×10^{-6} M). Fraction V, containing 95 per cent albumin, used in the titrations. +, glycine, pH 9.9; □, borate, pH 9.0; △, phosphate, pH 7.5; ×, phosphate, pH 6.0; ●, acetate, pH 5.0.

TABLE I

Effect of Concentration on Dissociation of Methemalbumin at pH 7.4 and Ionic Strength 0.1

	Concentration, moles per liter $\times 10^6$, as albumin		
	3.85*	0.385	0.077
Extinction per cm. at 403 mμ	0.551	0.0523†	0.0102†
" " " corrected for dilution	0.551	0.523	0.510
" " " calculated (Equations 7 and 8)	0.555	0.537	0.520
Mole fraction of ferriprotoporphyrin as *M*	0.03	0.11	0.23
" " " " " *LM*	0.04	0.10	0.17
" " " " " *LMM*	0.93	0.79	0.60

* Dilutions were made of this solution which had a composition of 2 moles of ferriprotoporphyrin per mole of albumin. The calculated extinctions have been corrected for contamination of the ferriprotoporphyrin with 5 per cent of the non-binding impurity.

† These solutions were measured in a 10.0 cm. cuvette.

added in large excess. The acid dissociation was reversed when the pH was adjusted to 7.0 or above by the addition of an alkaline buffer.

When ferriprotoporphyrin was added to albumin solutions in phosphate buffers of different ionic strength at pH 7.4, differences in end-point were not observed when the ionic strength varied from 0.03 to 0.30.

These experiments suggest that methemalbumin formation varied with the pH and with the specific composition of the buffers from pH 4.5 to 7.0. From pH 7.0 to 10.0, however, maximal binding appears to have occurred and to have been independent of pH and ionic strength.

Influence of Concentration—When a solution of methemalbumin was diluted, it failed to obey Beer's law. The deviation suggested the dissociation of the complex LMM in dilute solution to yield increased amounts of the species LM and M. The results are presented in Table I. The calculated extinctions and mole fraction of ferriprotoporphyrin as M, LM, and LMM at the given concentrations have been obtained by use of the equations and the intrinsic association constant, K_0, which are discussed below.

DISCUSSION

Since the experimental methods used did not permit direct measurement of the molecular concentrations of the various species involved in the equilibrium, experimental equilibrium constants could not be calculated. In an attempt to assess the magnitude of the constants, we have made use of equations applicable to such equilibria. This treatment has been used for the dissociation of carbonic acid and is similar to that used by Michaelis and Schubert (10) for a two-step oxidation-reduction, and by Shack and Clark (5) for metalloporphyrin coordination.

Using the symbols of Clark, with L representing the ligand, serum albumin, and M the metalloporphyrin, one obtains two equilibrium constants, Equations 3 and 4, for the reactions expressed by Equations 1 and 2.

$$\frac{[LM]}{[L] \times [M]} = K_1 \tag{3}$$

$$\frac{[LMM]}{[LM] \times [M]} = K_2 \tag{4}$$

At any point in a titration, the total concentration of serum albumin [1] whether free or combined, and of ferriprotoporphyrin [m] in all forms, is given by the expressions

$$[1] = [L] + [LM] + [LMM] \tag{5}$$

$$[m] = [M] + [LM] + 2[LMM] \tag{6}$$

By combining the four equations, Nos. 3 to 6, and introducing the molecular extinction coefficient (ϵ) of the various absorbing species, one arrives at a pair of equations, Nos. 7 and 8, which serve to define the optical density, D, of a solution, 1 cm. in depth, as a function of the concentration of ferriprotoporphyrin added at each step of the titration.

$$D = \frac{\epsilon_L + \epsilon_{LM} K_1[M] + \epsilon_{LMM} K_1 K_2 [M]^2}{1 + K_1[M] + K_1 K_2 [M]^2} \times [1] + \epsilon_M [M] \tag{7}$$

$$[m] = \frac{K_1[M] + 2K_1 K_2 [M]^2}{1 + K_1[M] + K_1 K_2 [M]^2} \times [1] + [M] \tag{8}$$

In the above equations, ϵ_L was zero, since a protein blank was used. ϵ_M varied slightly with protein concentration, owing to the non-specific effect of protein. In the absence of protein, $\epsilon_M = 3.65 \times 10^4$ (403 mμ); at 0.05 gm. of albumin per liter, $\epsilon_M = 3.9 \times 10^4$; and at 0.50 gm. of albumin per liter, $\epsilon_M = 4.2 \times 10^4$. Interpolation was used for intermediate values. The experimentally determined values for ϵ_{LM} and ϵ_{LMM} were used; namely, $\epsilon_{LM} = 8.3 \times 10^4$ and $\epsilon_{LMM} = 15.0 \times 10^4$.

In a recent discussion of the interaction of small molecules and ions with proteins, Scatchard (11) has shown that the experimental results can often be described by assuming that the protein possesses a maximum of n sites which bind the small molecule. In many instances, all n points can be assumed to have the same intrinsic association constant. The formation of any given protein-small molecular compound, in which the number of small molecules is generally less than n, will be described by this constant, modified by statistical and electrostatic factors. To describe the interaction of albumin with ferriprotoporphyrin we have made two assumptions: that $n = 2$ and that the reaction at either point is independent of combination at the other, except for statistical effects. Then, if we denote the intrinsic association constant by K_0, the actual equilibrium constants K_1 and K_2 in the equations above are related to K_0 as follows:

$$K_1 = \frac{n}{1} K_0 = 2K_0 \tag{9}$$

$$K_2 = \frac{n-1}{2} \times K_0 = \tfrac{1}{2} K_0 \tag{10}$$

$$\frac{K_1}{K_2} = 4 \tag{11}$$

The results obtained from the titrations at a wave-length of 623 mμ and the interaction of ferriprotoporphyrin with protein which we have termed non-specific may indicate that there are other sites on the albumin molecule which react with ferriprotoporphyrin. However, the formation of these higher compounds had little or no effect upon spectral absorption at the

403 mμ wave-length, and the association constants for their formation must be much smaller than that for the addition of the first two groups. Therefore, the formation of such higher complexes should not materially invalidate the assumption that a single intrinsic association constant can be used for the first two groups.

Based on the above considerations, the attempt was made to choose an intrinsic association constant by the construction of a set of titration curves. Good agreement with experimental data was obtained by use of the single association constant $K_0 = 10^8$, where $K_1 = 2 \times 10^8$ and $K_2 = 5 \times 10^7$. The titration curves so obtained are represented in Figs. 3 and 5. The calculated extinctions at the three levels of concentration shown in Table I also agreed well with the experimental values. Since dilution mainly affected the second equilibrium, these results tend to substantiate the value assigned to K_2.

While the binding of ferriprotoporphyrin by albumin can be satisfied by a single intrinsic association constant, K_0, according to the above treatment, it should be emphasized that this is not an experimentally derived constant. Indeed, good agreement was also obtained when $K_1 = 10^9$ and $K_2 = 10^7$ ($K_1/K_2 = 100$). It is, therefore, not possible to make any deductions from these data as to the extent of interaction, if there is any, between the two heme groups on the albumin molecule.

A large body of previous data (5) indicates that ferriprotoporphyrin exists in solution as large micelles within which there are dimeric units. Although in the development of our equations the metalloporphyrin has been treated as a monomeric species, exactly the same titration curves are obtained on the basis of dimeric units. The equations merely indicate the molecular proportions of the various components and do not reveal the degree of polymerization.

No observable differences in behavior toward ferriporphyrin were found with the several lots of human serum albumin used. Albumin alone, of the plasma proteins studied, interacted with ferriprotoporphyrin in this way. Preliminary observations with crystalline bovine serum albumin suggested that its ferriprotoporphyrin complex was different in spectral characteristics from the complex with human serum albumin. The studies with other plasma proteins and a method for the quantitative determination of albumin based on the present investigation will soon be reported.

This study supports the validity of measurements previously reported (1) on plasma methemalbumin levels during the administration of certain drugs. Assuming an average of 35 gm. of serum albumin per liter of plasma, over 300 mg. of hemin per liter of plasma could be carried in the form of the 1:1 complex. The highest levels encountered were 25 mg. per liter, and this would combine with less than 10 per cent of the serum albumin. Under such conditions, all the ferriprotoporphyrin would be bound. Furthermore, dilution of methemalbumin-containing plasma for measurement was never greater than 5-fold, and this would have resulted in no appreciable dissociation of the complex.

We thank Miss Elizabeth O. Burr for assistance in these measurements.

SUMMARY

Spectrophotometric titration at the 403 mμ absorption band of methemalbumin indicated that human serum albumin reacts with ferriprotoporphyrin IX to form protein-heme complexes in the molar ratios of 1:1 and 1:2. By use of the mass law equations, association constants have been chosen which give good agreement with the experimental results.

Reversible dissociation of the complex occurred between pH 7.0 and 4.6; between pH 7.0 and 10.0 the reaction was not affected by pH, specific ion effects, or moderate changes in ionic strength. The reaction was accelerated at 38° and equilibrium was established within ½ hour at this temperature.

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BIOCHEMICAL STUDIES ON CHLORAMPHENICOL (CHLOROMYCETIN*)

III. ISOLATION AND IDENTIFICATION OF METABOLIC PRODUCTS IN URINE†

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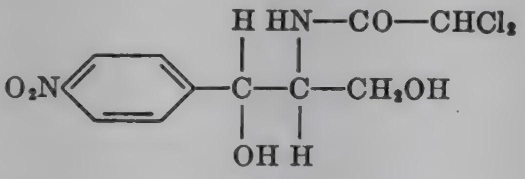
The antibiotic chloramphenicol (Chloromycetin), first isolated from cultures of *Streptomyces venezuelae* (1), has been identified as D(−)-*threo*-1-*p*-nitrophenyl-2-dichloracetamido-1,3-propanediol (2). The structure of this compound is shown below:



(I)

When chloramphenicol is administered by mouth to normal human subjects, only 5 to 10 per cent of the administered dose is excreted unchanged in the urine, as determined by microbiological assay methods (3, 4). However, with colorimetric methods for aromatic nitro compounds (5), from 75 to 90 per cent of the administered dose is accounted for in 24 hour urinary excretion in man (3). The major portion of these nitro compounds exhibits no antibiotic activity, and represents inactive degradation products of chloramphenicol. In lower animals, lesser amounts of nitro compounds are recovered in the urine, the remainder being excreted in the bile and feces, where considerable reduction occurs with the formation of aromatic amines (3).

The present communication is concerned with the isolation and identification of chloramphenicol and its metabolic products from urine, chromatographic and counter-current methods for separation being used. The major excretory product in urine is shown to be a monoglucuronide derivative of chloramphenicol by enzymatic and chemical degradation studies.

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* Parke, Davis and Company's trade name for chloramphenicol.

† Reported in part at the thirty-third annual meeting of the Federation of American Societies for Experimental Biology, Detroit, April (1949).

Procedure

Aromatic nitro compounds are determined colorimetrically by reduction with titanous chloride, followed by diazotization and coupling with the Bratton-Marshall reagent (5). The results are expressed as "chloramphenicol equivalents," since metabolic products of chloramphenicol containing the nitro group, but having no antibiotic effect, are included in the analysis. Chloramphenicol is usually determined by microbiological assay with *Shigella sonnei* by using a turbidimetric procedure (6), although the colorimetric method is also satisfactory when the antibiotic is first separated from inactive nitro compounds by extraction with organic solvents (5).

Paper strip partition chromatography was conducted according to the methods described by Consden, Gordon, and Martin (7). The materials to be chromatographed were first concentrated in a narrow band on the paper strip (Schleicher-Schüll No. 112) by use of the hot wire technique described elsewhere (8). After chromatography the position of the nitro compounds on the strips was established by the formation of colored derivatives. With a fine atomizer a 1:10 dilution of the stock 15 per cent titanous chloride reagent in 1 N HCl is sprayed onto the paper strip. While still damp, the strip is lowered into a glass cylinder containing a small amount of liquid bromine at the bottom, and the excess titanous reagent is rapidly oxidized by the bromine vapor, eliminating any interference with subsequent reactions. The treatment with bromine seems to affect the reduced compounds directly, since the carmine color resulting from this procedure is quite different from the amethyst color produced in the usual colorimetric procedure without bromination (5). The strip is then aired in a hood to remove the free bromine. After a few minutes, the strip is sprayed with a mixture of 10 parts butyl nitrite, 5 parts *n*-butanol, and 1 part glacial acetic acid. After standing 2 minutes, the strip is sprayed with a 0.1 per cent solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride in a mixture of 95 ml. of *n*-butanol and 5 ml. of 2 N HCl. A pink color indicates the presence of aromatic nitro compounds. A control for aromatic amines is also run by spraying the control strip with butyl nitrite and the coupling reagent, but omitting the reduction and bromination steps.

EXPERIMENTAL AND DISCUSSION

Counter-Current Partition of Nitro Compounds in Rat Urine—Since the administration of chloramphenicol has been shown to result in the excretion of more than one aromatic nitro compound in the urine (3), an attempt was made to fractionate these compounds by the counter-current extraction procedures described by Craig (9). A 24 plate apparatus was used in these experiments. The solvents selected gave approximately equal distribution of chloramphenicol between equal volumes of the two immiscible phases at 24°. These consisted of 0.2 M phosphate buffer at pH 6.7 and a mixture of 79 per cent heptane and 21 per cent isoamyl alcohol. The aqueous and organic phases were mutually saturated by shaking together before use.

Urine collected from a series of rats over a 17 hour period following subcutaneous administration of 100 mg. of chloramphenicol per kilo, body weight, was diluted with an equal volume of 0.4 M phosphate buffer at pH 6.7. 8 ml. of this mixture were extracted in the 24 plate Craig apparatus against 8 ml. of the heptane-isoamyl alcohol mixture described above. The aqueous and organic phase in each plate was then analyzed for aromatic nitro compounds in terms of chloramphenicol equivalents by ultraviolet absorption at 278 mμ. A parallel series was set up in the same manner by using a known sample of chloramphenicol. The results are presented in Fig. 1.

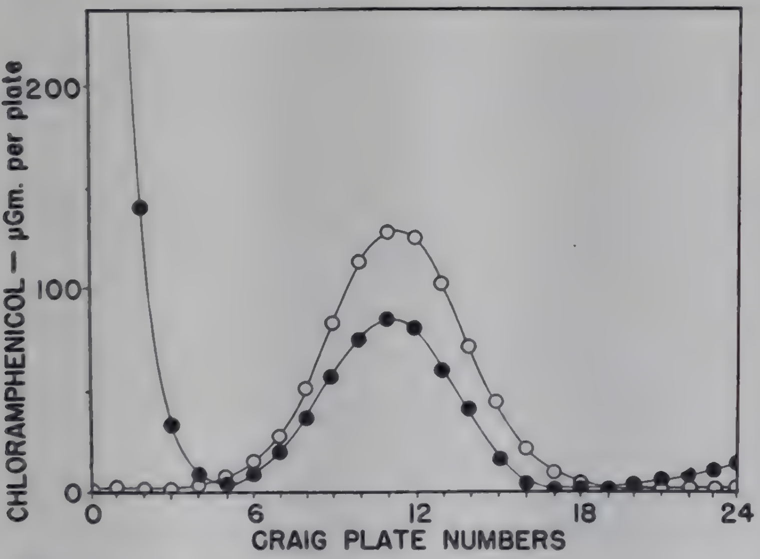


FIG. 1. Counter-current extraction of rat urine to demonstrate the presence of chloramphenicol. ○, obtained with a known sample of chloramphenicol; ●, obtained with rat urine. The water-soluble end of the series is in the lower plate numbers.

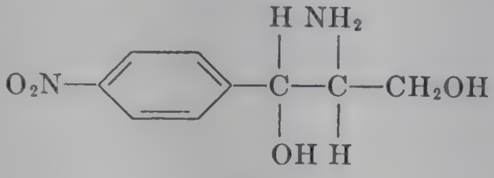
From these data it is evident that a high concentration of a water-soluble nitro compound is present in the urine. A second component in the middle of the series (with peak concentration at Plate 11) corresponds in distribution with the known sample of chloramphenicol. The substance in Plate 11 showed the same maximum ultraviolet absorption as observed with chloramphenicol (278 mμ), further establishing its identity.

Isolation of Chloramphenicol from Human Urine—The presence of unchanged chloramphenicol in human urine was definitely established by direct isolation and crystallization of the antibiotic. Urine specimens were obtained from patients undergoing treatment with chloramphenicol for bacillary infections of the urinary tract. Approximately 4 liters of urine at pH 5 were extracted with 1500 ml. of ethyl acetate, and extracted a second time with 1000 ml. of the organic solvent. The ethyl acetate extracts were pooled and evaporated to dryness under reduced pressure at room temperature. The residue was further purified by an 8 plate countercurrent extraction with 0.2 M phosphate buffer at pH 6.7 and a mixture of 79 per cent heptane and 21 per cent isoamyl alcohol. This extraction procedure was carried out in 500 ml. glass-stoppered Pyrex bottles, with 150 ml. volumes of phosphate buffer and the heptane-isoamyl alcohol mixture described above. The solutions in Plates 3, 4, and 5, containing most of the chloramphenicol, were pooled and concentrated by evaporation. The residue was extracted with three separate portions of ethyl acetate, the pooled extracts were evaporated to dryness, and the residue was redissolved in chloroform. On the addition of 6 volumes of petroleum ether a heavy precipitate appeared. The precipitation process from chloroform was repeated several times and the final product was dried in an oven at 100°. This preparation gave a melting point of 151.0° as compared with 151.5°, for a known sample of chloramphenicol (uncorrected readings), and produced no depression in melting point when mixed with chloramphenicol. Maximum ultraviolet absorption was found at 278 mμ, and the $E_{1\,\text{cm.}}^{1\%}$ value was found to be 290 in 0.1 N HCl and 293 in pH 6.6 phosphate buffer, compared with values of 290 to 297 obtained with known samples of chloramphenicol. When assayed for antibiotic activity, this preparation was found to possess 98 to 100 per cent of the activity of a known sample of chloramphenicol.

Paper Strip Chromatography of Urine—Paper strip partition chromatography was used to study the metabolic products of chloramphenicol in urine. The solvent employed was a mixture of *n*-butanol, with 2.5 per cent phenol and 2 per cent pyridine added. Nitro compounds were detected by the color reaction already described ("Procedure"). Typical data obtained with urine specimens after administration of chloramphenicol are presented in Table I. Urine from man, dog, and rat showed the presence of three principal aromatic nitro compounds. These were identified by comparing the R_F values with known derivatives of chloramphenicol and appeared to be the same in all the species studied.

Band I was found to be identical with that produced by the addition of chloramphenicol to normal urine. It represents the small amount of antibiotic excreted in the urine following the administration of this drug, whose isolation and chemical identity with chloramphenicol were established in the preceding section.

Band II was found to correspond with that produced by the addition of D(−)-*threo*-1-*p*-nitrophenyl-2-amino-1,3-propanediol to normal urine. This compound is readily obtained by acid or alkaline hydrolysis of chloramphenicol at the amide linkage with the formation of dichloroacetic acid and the optically active base (2). Its structure is given below:



(II)

In all urine specimens studied Band III was most prominent, giving the greatest color intensity and covering the greatest area. The band due to chloramphenicol (I) was less marked, while that due to the amino diol hydrolysis product (II) was least distinct. Consequently it would appear that the substance responsible for Band III is the principal metabolic product of chloramphenicol excreted in the urine of man, dog, and rat. The position of Band III indicates that it is more water-soluble than either chloramphenicol or its hydrolysis product, and it probably corresponds with the water-soluble nitro compound which was noted in the counter-current extraction experiment (Fig. 1).

TABLE I

Chromatography of Nitro Compounds in Urine Following Administration of Chloramphenicol

Urine samples were chromatographed on paper strips by the descending technique, and the nitro compounds were detected by the color reaction described in the text. In each experiment known samples of chloramphenicol, the base resulting from the hydrolysis of chloramphenicol, and chloramphenicol-glucuronide were added to normal urine samples to serve as controls.

Sample chromatographed	Band I	Band II	Band III
	R_F	R_F	R_F
Urine, rat	0.89–0.94	0.54–0.60	0.30–0.37
" dog	0.90–0.95	0.58–0.63	0.34–0.40
" man	0.92–0.95	0.58–0.65	0.28–0.32
Chloramphenicol (I)	0.89–0.95		
Base from hydrolyzed chloramphenicol (II)		0.56–0.67	
Chloramphenicol-glucuronide (III)			0.30–0.40

Similar results were obtained with two-dimensional chromatographic methods. In all cases controls were run by the addition of known compounds to the urine specimens. The first solvent employed was *n*-butanol containing 1.5 per cent glacial acetic acid, while the second solvent was *n*-butanol with 1.5 per cent ammonium hydroxide used. Colors were developed following reduction with titanous chloride, as already described for the paper strips. By using samples of rat and dog urine collected after parenteral administration of chloramphenicol, three distinct spots were observed, corresponding to Bands I to III on the single dimensional strips. In addition, two other faint spots were observed, one being close to the area due to chloramphenicol and the other close to the substance responsible for Band III.

The chromatographic technique was also applied to the study of nitro compounds in bile collected from dogs and guinea pigs. Although high concentrations of nitro compounds have been observed in bile, the antibiotic activity is usually quite low (3). By using the paper strip partition procedure described above, the predominant nitro compound in bile was found to have the same R_F value as the substance in urine characterized by Band III.

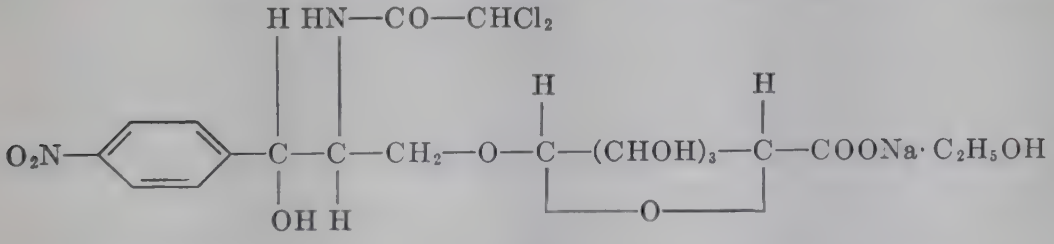
Isolation and Identification of Glucuronide Derivative of Chloramphenicol from Urine—The compound responsible for Band III on the paper strip chromatograms was isolated by the procedure described below, and identified as a monoglucuronide derivative of chloramphenicol.

6 gallons of urine collected from human subjects who were undergoing treatment with chloramphenicol for urinary tract infections were concentrated to 3500 ml. in a vacuum pan at room temperature and filtered. The salt content of the filtrate was decreased by freezing the mixture until an icy slush formed and removing the solids by centrifugation. An equal volume of *n*-butanol was then added to the urine concentrate (at pH 6.7) and an 8 plate counter-current extraction series was run with Pyrex bottles instead of with the Craig apparatus (9). At the end of the run, petroleum ether was added to each plate equivalent to two-thirds the volume of the organic phase, and on shaking, the nitro compounds were found to be completely transferred to the aqueous phase. Aliquots were analyzed for nitro compounds by the titanium reduction procedure (5), and the highest concentrations were found close to the middle of the series.

The three central plates containing the highest concentrations of nitro compounds were pooled and concentrated in a vacuum pan. The pH was adjusted to 8.0 and the solution cooled in a deep freeze unit to crystallize out excess salts. After readjustment of pH 6.7, the solution was subjected to a second counter-current extraction under the conditions already described. The contents of the central plates were again pooled and concentrated in a vacuum pan. The concentrate was then lyophilized to remove the water and redissolved in absolute ethanol. The addition of petroleum ether resulted in the appearance of a precipitate which was filtered off, washed with excess petroleum ether, and dried. The material was again dissolved in absolute ethanol and reprecipitated with petroleum ether.

The final preparation was a fine white powder which proved to be extremely hygroscopic, turning brown in the presence of moisture. It was dried to constant weight *in vacuo* over P_2O_5 in 24 to 48 hours at room temperature. When heated, it decomposed without giving a sharp melting point. The compound was extremely soluble in water, soluble in the lower alcohols, slightly soluble in acetone, and insoluble in ether. Qualitative tests revealed the presence of chlorine and an aromatic nitro group. A faint positive test was obtained for glucuronic acid by the naphthoresorcinol reaction, and infra-red absorption data supported this observation. The ultraviolet absorption in methanol showed a peak at 273 mμ, presumably due to the para-nitro structure, with an $E_{1\,\text{cm.}}^{1\%}$ of 167. The apparent molecular weight calculated from the ultraviolet absorption data and corrected for volatile loss is 566.

Microanalytical data gave the following per cent composition: C 40.48, 40.37; H 4.48, 4.73; N (Dumas) 5.03, 5.03; total Cl 11.56; Na 4.98; O (by difference) 33.44; ash 8.31. Based on a molecular weight of 566, the following empirical formula is obtained: $C_{19}H_{25}N_2Cl_2O_{12}Na$. This is in fair agreement with the following structure, representing a monoglucuronide derivative of chloramphenicol with one alcohol of crystallization present:



(III)

This compound has a molecular weight of 567, being represented by the empirical formula $C_{17}H_{19}N_2O_{11}Cl_2Na \cdot C_2H_5OH$. The theoretical per cent composition is C 40.20; H 4.41; N 4.94; Cl 12.51, Na 4.05; O 33.84, showing good agreement with the experimentally determined composition. The actual point of attachment of glucuronic acid to chloramphenicol was established by the experiments described in another section. The compound appeared to be homogeneous, yielding a single nitro band on paper chromatography and giving a single peak in the center of the distribution series when subjected to a counter-current separation in the 24 plate Craig apparatus between 0.2 M phosphate buffer at pH 6.7 and *n*-butanol.

Enzymatic Hydrolysis of Chloramphenicol Glucuronide—The compound described in the preceding section showed no antibacterial activity when tested against *Shigella sonnei*. Its probable structure (III) indicates that chloramphenicol is in no way altered, except for conjugation with glucuronic acid through a single —OH group. Consequently attempts were made to liberate active chloramphenicol by enzymatic hydrolysis. It was found that more than 50 per cent of the theoretical activity could be regained by incubating this conjugate with minced rat liver or spleen, and that the optimum pH for this hydrolysis was 4.5 to 5.0. Purified preparations of β-glucuronidase gave even better recovery of active antibiotic, often exceeding 70 per cent of the theoretical chloramphenicol content.

Several preparations of β-glucuronidase were made by the methods described by Graham (10) and Fishman (11). One of the latter preparations contained 2.34 mg. of N per ml. by micro-Kjeldahl analysis, and showed an activity of 4000 units per ml. by using a phenolphthalein glucuronide substrate (12). 2 ml. of this enzyme preparation were added to 120 ml. of 0.1 N acetate buffer at pH 5.0 containing approximately 15 mg. of the chloramphenicol glucuronic acid conjugate, and the mixture was incubated at 38°. At various time intervals aliquots of the solution were removed by pipette and heated in a boiling water bath for several minutes. This treatment was found to inactivate the enzyme without destroying chloramphenicol or hydrolyzing the glucuronide conjugate. The samples were filtered and analyzed for total nitro compounds by the titanium reduction method, and for unaltered chloramphenicol by the solvent extraction method and by microbiological assay. The results presented in Table II indicate that the antibiotic is reformed from the inactive conjugate. The hydrolysis appears to follow the kinetics of a first order reaction over the first 4 hour period. Under identical conditions with heat-inactivated enzyme added to the glucuronide, no antibiotic activity appeared.

Hydrolysates prepared in a similar manner were chromatographed on paper strips to demonstrate the presence of chloramphenicol and glucuronic acid. The nitro compounds were chromatographed in *n*-butanol containing 2.5 per cent phenol and 2.0 per cent pyridine, and colors were developed with the titanium-bromine diazotization reagents already described. The presence of glucuronic acid was established by chromatography with *n*-butanol containing 10 per cent acetic acid, and the bands were made visible by spraying with ammoniacal silver nitrate and heating, as described by Partridge (13). Controls were run with conjugate plus heat-inactivated enzyme, and with known preparations of chloramphenicol and sodium glucuronate added. It was found that bands corresponding to chloramphenicol and glucuronic acid appeared after enzymatic hydrolysis of the conjugate. A trace of reducing material was also observed in the enzyme preparation itself with an R_F of 0.30, but this did not interfere with the identification of glucuronic acid.

Isolation of Hydrolysis Products—The identification of chloramphenicol and glucuronic acid as hydrolysis products was confirmed by direct isolation of chloramphenicol, and by preparation of known derivatives of glucuronic acid.

A solution containing 800 mg. of chloramphenicol glucuronide in 85 ml. of 0.12 M acetate buffer at pH 4.5, was incubated with 15 ml. of a β-glucuronidase preparation for 44 hours at 39°. Microbiological assay showed that 59 per cent of the theoretical antibiotic activity had appeared. The pH of the solution was adjusted to 6.5 and was then extracted four times with equal volumes of ethyl acetate. The extracts were pooled and dried over anhydrous magnesium sulfate. The crystalline residue was dissolved in 5 ml. of hot water and recrystallized, yielding 177 mg. of crystalline material (72 per cent recovery). The compound melted at 149–150° and produced no depression of melting point when mixed with a known sample of chloramphenicol. The ultraviolet absorption and microbiological activity showed characteristics identical with those of chloramphenicol.

TABLE II

Hydrolysis of Chloramphenicol-Glucuronic Acid Conjugate with β-Glucuronidase

The glucuronide of chloramphenicol isolated from human urine was incubated with β-glucuronidase at pH 5.0 and 38°. All the results are expressed as micrograms of chloramphenicol equivalents per ml. of solution. The colorimetric method is used to determine total nitro compounds, while the solvent extraction and microbiological methods are used for active antibiotic compounds.

Time of incubation	Method of analysis			Liberated chloramphenicol
	Colorimetric	Microbiological	Solvent extraction	
min.	*µgm. per ml.*	*µgm. per ml.*	*µgm. per ml.*	*per cent*
5	62.1	1.5	1.1	1.8
10	61.5	2.4	2.0	3.3
20	63.9	4.7	4.1	6.7
40	62.5	9.1	8.3	13.5
80	62.0	17.4	15.5	25.2
160	62.6	32.0	27.4	44.6
242	62.3	39.0	34.0	55.2
615	61.2	43.0	41.9	68.2
1440	63.5	45.0	45.9	74.8
Control	61.9	<1	<1	0

The aqueous residue from the preceding extraction was then evaporated under reduced pressure to 18 ml. and dialyzed in cellophane casing against 200 ml. of distilled water overnight in the refrigerator. The dialysate was evaporated to 10 ml. under reduced pressure at 50°, and the resulting solution showed reducing activity by the Fishman modification of the Miller-Van Slyke procedure (11) close to the theoretical amount of glucuronic acid liberated by hydrolysis, and gave a positive color test for glucuronic acid by the naphthoresorcinol reaction.

An attempt was made to prepare a derivative of glucuronic acid which could be readily isolated and identified. The procedure used was similar to that described by Lohmar *et al.* (14), in which glucuronic acid is first oxidized to saccharic acid with bromine, and the insoluble dibenzimidazole derivative is then formed by condensation with *o*-phenylenediamine.

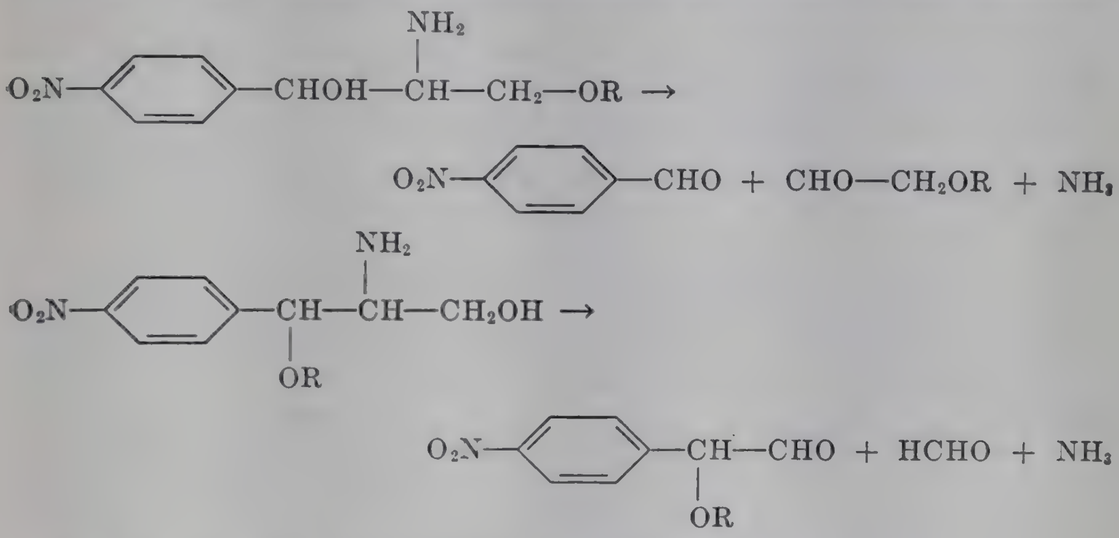
9 ml. of the dialysate concentrate described above were shaken with 1.45 gm. of barium benzoate and bromine at 0° for 2 hours, and then allowed to stand 70 hours at room temperature. The solution was then aerated to remove excess bromine, acidified with sulfuric acid, and the precipitate of benzoic acid removed by filtration. The filtrate was then treated with 0.33 gm. of silver carbonate in the dark for 2 hours, and the resulting solution was filtered, extracted three times with chloroform, and then evaporated to dryness. The residue was dissolved in acid and the solution treated with 280 mg. of *o*-phenylenediamine and 1 ml. of diethylene glycol at 135° for 2 hours. After decolorizing with norit, the pH was adjusted to 9 to 10 with ammonium hydroxide and the solution allowed to stand for 2 days. The solution was then acidified with HCl to pH 2 and extracted twice with ether and twice with ethyl acetate, following which it was made alkaline with ammonia and extracted three times with ether. The desired product was not extracted under these conditions. Upon seeding with less than 0.5 mg. of a known preparation of the dibenzimidazole of saccharic acid, the derivative crystallized.

The product was filtered off after 20 hours, yielding 58 mg. (m.p. 215–220°), with no depression of the melting point of the known dibenzimidazole (14). Upon recrystallization from ammoniacal solution, a product was obtained with a melting point of 225–230°. No depression of melting point was observed when mixed with a purified sample of saccharic acid dibenzimidazole. The known compound showed a melting point of 225–230° at a corresponding stage of purification, and 235° after recrystallizing.

Comparative ultraviolet absorption data for the benzimidazole derivatives of gluconic and saccharic acids, as well as for the compound described above, are presented in Table III. Three absorption maxima were observed at 245, 273.5, and 280 mμ. The absorption characteristics of the unknown were closely parallel to those of saccharic acid dibenzimidazole, but the $E_{1\,\text{cm.}}^{1\%}$ values were somewhat lower, due to the presence of impurities.

The recrystallized material was also converted to the picrate by treatment with picric acid in hot aqueous solution. After several recrystallizations, the derivative was found to melt at 211–212°, with no depression of the melting point of the known picrate (14).

Degradation Studies with Periodic Acid—Theoretically glucuronic acid might be conjugated with chloramphenicol through the —OH groups in either the 1 or 3 position in the side chain. After liberating the free amine by alkaline hydrolysis, oxidation with periodic acid would be expected to yield *p*-nitrobenzaldehyde from the 3-glucuronide, whereas formaldehyde would result from oxidation of the 1-glucuronide, as shown below:



The simultaneous degradation of glucuronic acid might also be expected, but it was found that this side reaction could be minimized by controlling temperature and reaction time. By using 0.1 N to 1.0 N NaOH at room temperature for 20 hours, the alkali-treated glucuronide was found to take up approximately 0.8 equivalent more periodate than the control in 15 minutes at 0°, which is to be expected if only the amide linkage is being hydrolyzed. Since the strength of alkali does not appear to affect the results, it may be assumed that the glucosidic linkage is stable within these limits. However, oxidation for periods greater than 15 minutes resulted in increased uptake of periodate with time, presumably due to cleavage of the glucuronic acid fragment. Similar behavior was observed with a preparation of menthol glucuronide.

200 mg. of chloramphenicol glucuronide were dissolved in 9 ml. of 0.1 N NaOH and kept at room temperature for 20 hours. The solution was then neutralized with 5 N sulfuric acid and cooled to 0°. A solution of 10 ml. of ice-cold periodate (containing 200 mg. of $HIO_4 \cdot 2H_2O$ and neutralized with bicarbonate) was added. After 15 minutes at 0° the reaction mixture was extracted four times with equal volumes of ether. The ether extracts were evaporated and the residue was recrystallized from 2 ml. of hot water, yielding 26.1 mg. of crystalline material. The product

was identified as *p*-nitrobenzaldehyde with a melting point of 104–106° and showed no depression of melting point when mixed with the known compound.

The aqueous residue was then treated with 0.5 ml. of 2 M arsenite and 0.4 ml. of 20 per cent KI. After 15 minutes, the pH was adjusted with acetic acid to 4 to 5 and 1.5 ml. of dimedon reagent were added (100 mg. per ml. of absolute ethanol). A precipitate began to form in 5 minutes and was filtered off after 3 hours. On recrystallizing from 1 ml. of 50 per cent methanol, 9.3 mg. of solids were obtained which melted at 186–188° and showed no depression of melting point when mixed with a known sample of formaldehyde dimedon.

Controls were run with the amino diol (II) to establish recovery figures for the oxidation products. With 85 to 90 per cent recovery of *p*-nitrobenzaldehyde, the corrected yield from the glucuronide is 61 to 65 per cent. With a recovery of 70 per cent for the formaldehyde dimedon, the corrected yield is 14 to 18 per cent. The discrepancies in yield may perhaps be accounted for by the presence of impurities or by a slower rate of oxidation with periodic acid. However, it would appear that the principal form of the chloramphenicol-glucuronic acid conjugate is the 3-glucuronide, while the occurrence of a small amount of 1-glucuronide has not been ruled out.

TABLE III

Ultraviolet Absorption Characteristics of Hexuronic Acid Derivatives

$E_{1\,\text{cm.}}^{1\%}$ values shown in the body of the table were calculated for the three absorption maxima observed in aqueous solutions.

Compound	Ultraviolet absorption maxima, $E_{1\,\text{cm.}}^{1\%}$		
	$\lambda = 245$ mμ	$\lambda = 273.5$ mμ	$\lambda = 280$ mμ
Gluconic acid monobenzimidazole	224	284	256
Saccharic " dibenzimidazole	334	417	387
Derivative prepared from chloramphenicol conjugate	300	399	354

We are indebted to many individuals for their kind assistance at various stages of this work. Urine specimens were obtained from hospitalized patients through the courtesy of Dr. George Chittenden and Dr. Elwood Sharp. Dr. Gertrude Rodney gave valuable assistance in the preparation of enzymes, and numerous assays were made by Miss Loretta Wolf and Mrs. Margaret Galbraith. The ultraviolet and infra-red absorption data were obtained by Dr. John M. Vandenbelt, and the elementary analyses were performed by Mr. Charles Childs.

We are particularly indebted to Dr. A. Calvin Bratton, Jr., Dr. Harry M. Crooks, Jr., and to Mr. Leonard Doub for their helpful criticism and suggestions throughout the course of this work.

SUMMARY

Following the administration of chloramphenicol, the principal nitro compounds found in the urine of rat, dog, and man are (I) unchanged chloramphenicol, (II) a hydrolysis product of chloramphenicol, and (III) a conjugate of chloramphenicol with glucuronic acid. Of these, only chloramphenicol exhibits significant antibiotic activity.

Chloramphenicol and its conjugate with glucuronic acid have been isolated from human urine and identified. The glucuronide is readily hydrolyzed with the enzyme β-glucuronidase, yielding unchanged chloramphenicol and glucuronic acid. The hydrolysis products have been identified by paper partition chromatography, by direct isolation of chloramphenicol, and by the preparation of saccharic acid dibenzimidazole from glucuronic acid. The structure of this glucuronide is further established by degradation studies with periodic acid.

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ERYTHROCYTE PYROPHOSPHATASE IN HEALTH AND DISEASE

II. REVERSIBLE AND IRREVERSIBLE INACTIVATIONS OF THE ENZYME

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It was previously reported (1) that erythrocyte pyrophosphatase is greatly inactivated by incubation with M/35 veronal-acetate buffer for 30 minutes prior to assay, and that the presence of magnesium ions affords a certain amount of protection from such inactivation. Jacobsen (2) reported that the inorganic pyrophosphatases of liver, kidney, and bone were completely destroyed by warming for 5 minutes at 55°. That inorganic pyrophosphatase is highly labile was contended by Pillai (3) working with muscle. In view of such contradictory findings on the nature of the enzyme from various tissues it was thought worth while to study the rapid inactivation of the erythrocyte enzyme in more detail.

Since the protective action of Mg ions on the enzyme against what was regarded as heat inactivation, and since the enzyme requires free SH groups for its action, it was assumed that the inactivation observed with buffer alone might be due to the oxidation of thiols, resulting in a decrease in the adsorptive capacity of the enzyme, which is thus rendered incapable of reacting with the magnesium or more probably with the magnesium pyrophosphate molecule. That magnesium protects other thiol enzymes from inhibitions produced by oxygen was indicated by Dickens (4) who suggests that magnesium forms a complex with the SH groups. Several workers (4–6) have found that oxygen inactivates thiol enzymes. In addition to the inactivation of the enzyme by incubation for ½ hour with buffer, it was also found that the enzyme could be inactivated by bubbling oxygen gas slowly through the buffered enzyme solution for 15 minutes at 28°.

Considering the above two types of inactivation, one has to assume that the enzyme has essential groups, presumably thiols, which are so highly susceptible both to small changes of temperature and to oxygen that spontaneous inactivation might take place. But, on the other hand, it was found that, though the aqueous enzyme is gradually inactivated at room temperature, it retains more than 50 per cent of its original activity even after 6 hours at 28°. Further, the undiluted hemolysate, *i.e.* 1:10 erythrocytes, when incubated at 38° for 15 minutes suffered only negligible inactivation (sometimes a slight activation too was observed).

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Experiments were therefore repeated with water instead of buffer, to ascertain whether the buffer takes any part in the above inactivations, and it was found that in both cases the effect was slight when compared to that produced with buffer. It was thus recognized that the veronal-acetate buffer contributes to the major part of the inactivation. The results of the study of the inactivation by the buffer constituents and substances related to them, the reversibility of the inactivations, and protection against the inhibitors are detailed in this paper.

EXPERIMENTAL

Standard Assay—The procedure for the assay of the enzyme is the same as that given previously (1) except that the incubation period was reduced from 30 to 15 minutes.[1]

Effect of Buffer on Enzyme—It is well known that not only the pH optimum of an enzyme varies, but also that the extent of enzyme action is specifically influenced by different buffers. Wisansky (7) observed that invertase action is depressed when the concentration of the neutral buffer acid (acetate) at the same optimum pH is increased. Hence the inhibitory action of the buffer acids on the pyrophosphatase might be anticipated.

The pyrophosphatase has the definite advantage in having an optimum pH near the neutral point, and it exerts an enzyme action at pH 7.0 which is as much as 75 to 80 per cent of that at the optimum pH. Thus in an unbuffered aqueous medium (pH 7.0) the activity is 82 per cent in one case and 77 per cent in another (Table I). Table I also shows the marked inhibitory effects of the buffer acids compared to the slight inhibition with water.

However, prolonged incubation with water also produces marked inactivation, as is shown in Table II. The pyrophosphate ion seems to be a more efficient protector than the magnesium ion.

Inhibition Experiments

In order to find out whether the inhibition was due to any specific effect of either of the buffer constituents, further experiments were set up. It

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[1] Unless otherwise stated the incubation mixture has a final volume of 5 ml. at pH 7.6, with a final concentration of 0.02 M magnesium chloride and 0.001 M sodium pyrophosphate. Buffer quantities are varied as indicated in each case. 2.5 ml. of 10 per cent trichloroacetic acid were used for deproteinization. 5 ml. portions of the filtrate are always used for the estimation of the orthophosphate with a series of suitable standards. Blanks are set up simultaneously. As far as possible the experiments are run in duplicate. In many cases the duplicates agreed within 5 per cent, and whenever they differed by more than 10 per cent the experiments were repeated.

TABLE I

Influence of Veronal-Acetate Buffer on Erythrocyte Pyrophosphatase

In Experiment 1 the enzyme is directly assayed by adding 0.5 ml. of the hemolysate (1:10 erythrocytes) to a mixture of 3 ml. of M/35 veronal-acetate buffer or water, 1 ml. of magnesium chloride (0.1 M), and 0.5 ml. of 0.01 M sodium pyrophosphate, brought to 38°. In Experiment 2, 0.5 ml. of the enzyme is incubated with 3 ml. of M/35 veronal-acetate buffer (pH 7.6) or 3 ml. of water (pH 7.0) for 15 minutes at 38°, and then treated with magnesium and pyrophosphate as in Experiment 1, and the enzyme assayed.

Experiment No.	Enzyme activity	Activity as per cent standard	
		Potent enzyme	Weak enzyme
1	In buffer, pH 7.6	100	100
	" water, " 7.0	82	77
2	After incubation with buffer, pH 7.6, for 15 min. at 38°	17	<10
	After incubation with water, pH 7.0, for 15 min. at 38°	76	63

TABLE II

Protection of Enzyme by Pyrophosphate and Magnesium Ions

In Experiment 1, 0.5 ml. of the enzyme (1:10 erythrocytes) is treated with 3 ml. of M/35 veronal-acetate buffer, or with 3 ml. of buffer + 1 ml. of 0.1 M magnesium, or with 3 ml. of buffer + 0.5 ml. of 0.01 M pyrophosphate, and each incubated for 30 minutes at 38°. In Experiment 2, 5 ml. of the enzyme are treated with 10 ml. of magnesium, or 5 ml. of pyrophosphate + 5 ml. of water, or 10 ml. of water, and each incubated for 3 hours. The enzyme activity was measured in each case with 1.5 ml. portions after adding the requisite quantities of magnesium or pyrophosphate and bringing the volume to 5 ml. with buffer (2 ml.) and water. Assay period, 15 minutes; temperature, 38°; pH 7.6.

Experiment No.	Enzyme incubated with	Activity as per cent standard assay
1	Buffer alone	11
	" + pyrophosphate	51
	" + magnesium	32
2	Pyrophosphate	42
	Magnesium	29
	Water	<10

was found that acetate as well as veronal sodium was inhibitory. Not only the buffer constituents but substances related to them also produced inhibition.[2]

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[2] Regarding the molecular constitution and the accessibility to the enzyme, reference might be made to the exhaustive study of Quastel and Wooldridge (8) on dehydrogenating bacterial enzymes, and the work of Murray (9) on lipase.

From Figs. 1 to 3, it can be seen that luminal is more inhibitory than veronal. The inhibitory effect is more marked with weaker enzymes (Fig. 2), with which incubation with water alone produces about 20 per cent inhibition in 30 minutes. Further, it will be noted that the extent of inhibition with narcotics, as well as with malonate and acetate, increases with the length of incubation. Similar results were reported (10) on the action of narcotics on brain dehydrogenation.

Since malonic acid and urea form the two parts of the barbiturate structure, and since malonic acid is found to be the more inhibitory part (Fig. 3), it might be concluded that inactivation by narcotics was due to the malonyl radical. No doubt higher concentrations of urea produced inhibition, but with such high concentrations even Mg ions produced comparatively more effect. Since acetate is not so markedly inhibitory as the malonate, it might be that the carboxyl group exerts the inhibitor effect. The study was therefore extended to other carboxylic acids, the results of which are presented in Table III. The extent of the inactivation varied somewhat with the same inhibitor on different enzyme preparations, the weaker preparation becoming inactivated sooner. Hence, for purposes of

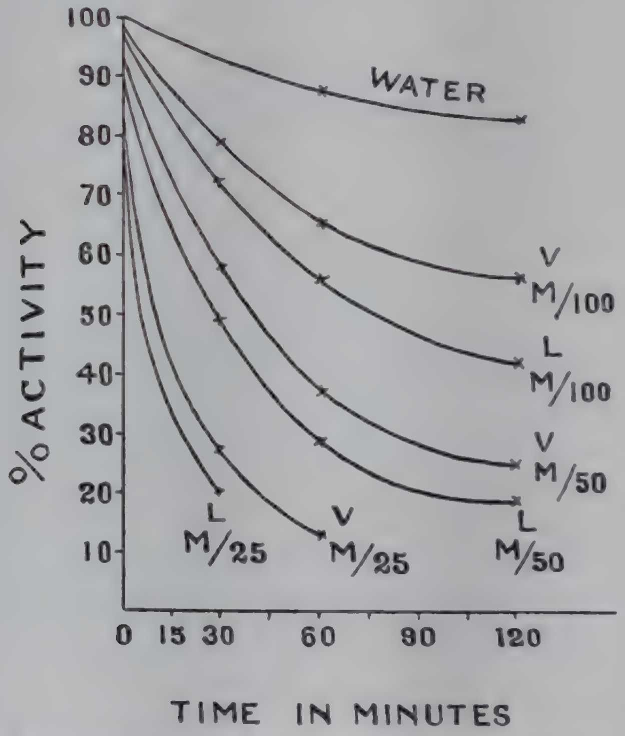

FIG. 1. Action of veronal and luminal. 0.5 ml. of the enzyme was treated with 3 ml. of water or the reagents of varying strengths at pH 7.0, and incubated at 38° for varying periods of time, and then assayed by adding 0.5 ml. of 0.01 M sodium pyrophosphate and 1 ml. of 0.1 M magnesium chloride. *L*, luminal sodium; *V*, veronal sodium.

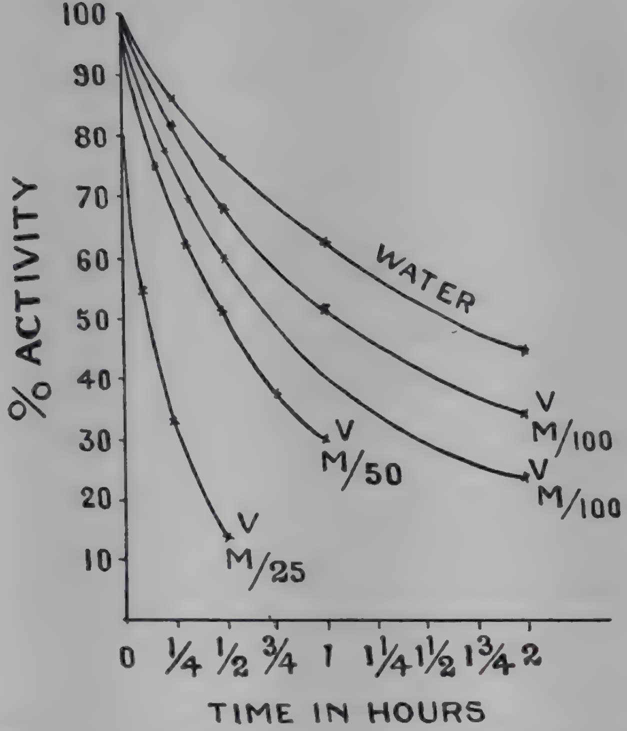

FIG. 2. Action of veronal. The experimental procedure is the same as is given below Fig. 1, but a weaker enzyme preparation was employed.

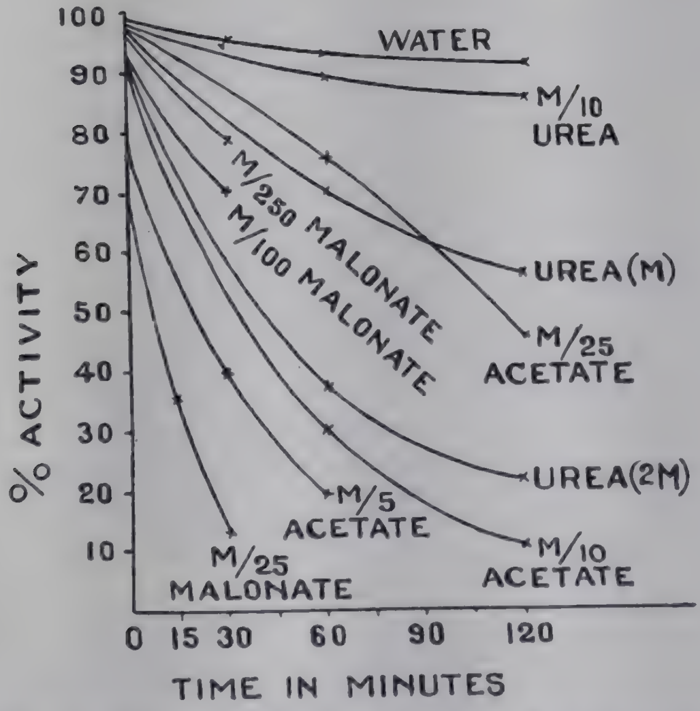

FIG. 3. Action of acetate, malonate, and urea. The experimental procedure is the same as given for Figs. 1 and 2, with a strong enzyme preparation.

comparison, the data obtained with different enzyme preparations of nearly the same potency are given in Table III.

Of the aldehydes formaldehyde is the most toxic. This inhibitory effect cannot be due to the reactivity of the carbonyl group alone, since in the carboxylic acids the carbonyl group is not so reactive as that in aldehydes or ketones. Almost the same result is observed with all the fatty acids tested as well as with benzoic acid. These results suggest that the car-

Table III

Inhibition of Erythrocyte Pyrophosphatase with Various Substances

0.5 ml. of the enzyme (1:10 erythrocytes) was incubated with 3 ml. of the inhibitor and adjusted to pH 7.0 for different time periods, as shown, at 38°. Controls incubated with water set up simultaneously. After the incubation the enzyme is treated with the requisite quantities of magnesium and pyrophosphate and assayed in unbuffered aqueous medium. Final volume, 5 ml.; period of hydrolysis, 15 minutes; temperature, 38°; pH 7.0. The per cent activity of the control and of the others was calculated, from which the per cent inhibition was noted by difference in each.

Inhibitor	Per cent Inhibition		Inhibitor	Per cent inhibition	
	Preliminary incubation period			Preliminary incubation period	
	15 min.	30 min.		15 min.	30 min.
0.2 M sodium acetate	60	80	0.1 M sodium glutamate		70
0.1 “ “ “	45	64	0.1 “ “ citrate	90	90
0.1 “ “ formate	43		0.1 “ “ adipate		71
0.1 “ “ propionate	40	65	0.1 “ “ benzoate		68
0.1 “ “ butyrate	48	65	0.1 “ glucose		5
0.1 “ “ chloroacetate	47		0.1 “ phenylalanine	8	
0.1 “ “ iodoacetate	90	90	0.1 “ tryptophan		80
0.1 “ acetamide	8	14	0.01 M “		15
0.1 “ glycine	6	11	0.01 “ tyrosine		72
0.1 “ alanine	4		0.005 M cystine		82
0.1 “ sodium pyruvate		90	0.1 M creatine		32
0.1 “ “ lactate		72	0.1 “ creatinine		8
0.1 “ “ mandelate		76	0.1 “ guanidine		90
0.1 “ “ oxalate	90	90	0.1 “ thiourea		90
0.1 “ “ malonate	90	90	0.1 “ urethane		31
0.1 “ “ succinate	30	48	0.1 “ formaldehyde	90	
0.1 “ “ aspartate	28	51	0.1 “ acetaldehyde		84
0.1 “ “ fumarate	90	90	0.1 “ benzaldehyde		36
0.1 “ “ maleate	90	90	0.1 “ semicarbazide		51
0.1 “ “ malate		90	0.1 “ sodium diethyl dithiocarbamate		81
0.1 “ “ tartrate		90			
0.1 “ “ glutarate		75			

boxyl group might be responsible for the inhibition. Replacement of the —OH in the —COOH with —NH_2 seems to decrease the inhibitor effect markedly (note acetamide in Table III). Also an electronegative group adjacent to the carboxyl seems to increase and an electropositive group like —NH_2 seems to annul the inhibitor effect. Thus glycine is non-inhibitory, and sometimes with lesser concentrations and shorter incubation periods it actually produced slight activation. In this connection it will be interesting to refer to the findings of Bodansky (11) who observed that glycine in higher concentrations inhibits bone and intestinal phosphatases and that the introduction of methyl groups into the amino group nullifies the inhibitor effect of the amino acid.

Malonate, oxalate, and citrate inhibition must be due to their greater number of carboxyl groups, but curiously enough, the dicarboxylate, succinate, is not even so inhibitory as acetate. However, when a double bond or hydroxyl groups are introduced, as in fumaric and maleic acids and malic and tartaric acids, there is greater inhibition than with succinate, while with the introduction of an amino group, as in aspartate, there is neither increase nor decrease in the inhibitor effect compared to succinate. Perhaps on account of steric effects only one carboxyl in succinate might be effective, whereas both the carboxyls might be effective in fumaric, maleic, malic, and tartaric acids. The finding (Table VI) that fumaric and malic acids are more inhibitory than maleic and tartaric acids, respectively, can be explained as due to steric hindrance. Similar steric effects due to different isomers of tartaric acids on acid phosphatase were noted by Abul-Fadl and King (12).

It will be observed that with propionate, pyruvate, lactate, and mandelate the hydroxyl group increases the inhibitor effect of the acid, and the keto group adjacent to a carboxyl group seems to impart more toxic properties. Similar conclusions were drawn by Dixon (13) regarding the types of lachrimators which inhibit thiol enzymes.

Creatine shows comparatively little inhibition, which is not the case with creatinine,[3] indicating that a free carboxyl group is probably necessary for inhibition, but such an assumption cannot be valid in the case of ascorbic and dehydroascorbic acids (not shown in Table III) in which the —COOH is not free. Further, substances containing hydroxyl groups cannot be inhibitive unless —COOH is also present. Thus glucose is practically non-inhibitory.

Acetone is not so inhibitory as acetaldehyde. Acetone, like urea, inhibits only in molar concentrations. *Substitution of the —S— or —NH— group for the —O— of urea bestows marked inhibitory properties.* Thus thiourea and guanidine are as strongly inhibitory as is malonic acid. Semicarbazide and diethyl dithiocarbamate caused inhibitions, while urethane produced only a slight effect. Both ethyl and methyl alcohols are inhibitory, but as the experiments were done under different conditions, the data are not shown.

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[3] In the case of creatinine the trichloroacetic acid filtrates should be diluted suitably before the phosphate is estimated.

Protection and Reactivation Experiments

To obtain more information on the nature of the reactions it was considered desirable to conduct experiments on reversal of the inhibitions. First of all the possible activators were tested.

It has already been reported (1) that bile salt is an activator. Cysteine and glutathione (GSH) were found to be good activators. Sodium sulfide and hyposulfite showed activation in some experiments, but unfortunately the sulfide especially also produced inhibition in some cases, particularly when the enzyme was kept in contact with them for longer periods. Their effects were not investigated further, and the data are therefore not presented. Cysteine is the most potent activator of all the substances tested. Different results are reported on the action of thiol compounds on various phosphatases (14–19), which are generally regarded as inhibitory. Roche *et al.* (20) found that intestinal pyrophosphatase (pH 7.8) is inhibited by cysteine, the inhibition disappearing in the presence of iodoacetic acid. They have also reported (21) that cysteine, GSH, Fe, Mn, and Zn produce a slight activation of liver pyrophosphatase (pH 7.4), and that the cysteine and Fe couple cause marked activation. The effect of thiols on the erythrocyte pyrophosphatase does not seem to have been investigated by them, though they found (22) activation of erythrocyte acid phosphatase by cysteine as well as by GSH.

It is found that cysteine is a strong activator of erythrocyte pyrophosphatase, and that the addition of Fe does not increase cysteine activation. Cysteine activation with fresh enzyme is not so marked as it is with old enzyme. Preliminary incubation with the activator for 15 minutes is necessary for maximum activation. Omission of this preliminary incubation sometimes produced negligible activation with fresh enzymes. Incubation at room temperature is preferable, since in a few cases the results were not so satisfactory when the incubation was carried out at 38°.

Vitamin C—Vitamin C is reported as an activator of certain phosphatases by some workers and as an inhibitor by others, while King and Delory (23) proved definitely that vitamin C has no action on serum alkaline phosphatase. Roche and others (20) have reported inhibition of intestinal pyrophosphatase (pH 7.8) with vitamin C, and working with mammalian erythrocytes (22), found a strong activation of acid pyrophosphatases (pH 3.8 and 5.8) with vitamin C. Maschmann and Helmert and Purr (24, 25) reported that papain is inhibited by vitamin C alone, but is activated by the vitamin C-Fe complex. It has been found that vitamin C is fairly inhibitory to erythrocyte pyrophosphatase (Table IV). The inhibition was high except in a few samples. At no time was any activation observed with either vitamin C or vitamin C-Fe complex. The latter is definitely inhibitory. The most interesting finding is that dehydroascorbic acid also is an inhibitor, and is more toxic than ascorbic acid in many cases. Thus the effect of ascorbic acid has no relation to its oxidation-reduction potential, and the inhibition must have been brought about by virtue of its structural configuration.

Table IV

Effect of Vitamin C and Cysteine on Enzyme

50 ml. of the hemolysate (1:10 human erythrocytes) were shaken with 5 ml. of chloroform and divided into two equal portions, of which one was kept in the refrigerator and the other at room temperature (28°). The next day, 0.5 ml. portions of both were treated with 0.5 ml. aliquots of the reagents, and, after 15 minutes standing at room temperature, the activities were determined by adding 2.5 ml. of buffer (pH 7.6), 1 ml. of 0.1 M magnesium chloride, and 0.5 ml. of 0.01 M pyrophosphate. The reagents, cysteine, and vitamin C were freshly prepared and aliquots added to the enzyme immediately after the reaction was adjusted to pH 7.6 with NaOH. Assay period, 15 minutes; temperature, 38°; pH 7.6.

	Enzyme kept at 4°		Enzyme kept at 28°	
	Per cent activation (+) or inhibition (−)	Per cent of original activity	Per cent activation (+) or inhibition (−)	Per cent of original activity
Enzyme alone		98		13
" + 1.58 mg. cysteine	+110	206	+136	31
" + 3.15 " "	+116	212	+255	46
" + 6.30 " "	+126	222	+318	55
" + 9.50 " "	+126	222	+373	62
" + 1.76 " vitamin C	−65	34	−100	0
" + 3.52 " " "	−86	12	−100	0
" + 5.28 " " "	−98	0	−100	0

Tables IV and V illustrate the results obtained with cysteine, cysteine-Fe complex, vitamin C, and vitamin C-Fe complex. Blanks were run for each experiment in these cases.

Protection Experiments

Table VI gives the results of protection of the enzyme with pyrophosphate and magnesium. 0.5 ml. of the enzyme is treated with 0.5 ml. of water, pyrophosphate, or magnesium, and then 0.5 ml. of the inhibitor (pH 7.6) is added to each, the mixture incubated at 38° for 30 minutes, and then the activity measured. A control without the inhibitor was also set up to assess the extent of inactivation produced by incubation with water alone. Two weak enzymes, E_1 and E_2, are employed for marked inhibition. With stronger inhibitors a relatively potent enzyme, E_3, is used.

The results in Table VI show that both pyrophosphate and magnesium are able to protect the enzyme almost completely except in the case of iodoacetic acid and guanidine. Even spontaneous inactivation due to water is prevented. The fact that the concentration of the pyrophosphate is a tenth that of the inhibitor suggests that the pyrophosphate is adsorbed at the active centers and thus blocks the inhibitors.

Table V

Effect of Vitamin C-Fe^{++} and Cysteine-Fe^{++} Complexes on Enzyme

An old enzyme, which has lost most of its activity, was used. 0.5 ml. portions of the enzyme were treated with 0.5 ml. of the reagents containing varying amounts of freshly prepared and neutralized solutions of cysteine, vitamin C, and ferrous ammonium sulfate. After 15 minutes standing at room temperature, the activities were measured as in Table IV.

Reagents added	Per cent activation (+) or inhibition (−)
Enzyme alone	
“ + 7.9 mg. cysteine·HCl	+165
“ + 7.9 “ “ + 0.39 mg. Fe^{++}	+117
“ + 7.9 “ “ + 0.19 “ “	+139
“ + 1.58 “ “ + 0.39 “ “	+41
“ + 1.58 “ “ + 0.19 “ “	+61
“ + 0.39 “ Fe^{++}	+13
“ + 0.39 “ “ + 2 mg. vitamin C	−100
“ + 0.19 “ “ + 2 “ “ “	−80
“ + 2 mg. vitamin C	−100

Since the concentration of Mg ions is greater than that of the inhibitor, it can be argued that the protection might be due only to combination of Mg with the inactivator, leaving no inhibitor to react with the enzyme. Such a possibility was recently discussed in another connection by Pardee and Potter (26). But in the present study one would expect, in such an instance, that the enzyme inactivated by incubation with the inhibitor should be capable of being reactivated with Mg. And since no such reversal could be achieved, it is feasible to assume that Mg and pyrophosphate have strong affinities for the active centers and thus are rapidly adsorbed; and, since the inhibitors act only gradually, they fail to produce inactivation when the enzyme is already saturated with Mg or pyrophosphate. Both pyrophosphate and Mg fail to protect the enzyme against certain inhibitors like copper, fluoride, guanidine, etc.

Table VI

Protection of Enzyme

0.5 ml. of the enzyme was treated with 0.5 ml. of water or 0.01 M pyrophosphate or 0.1 M magnesium, and then with 0.5 ml. of the inhibitor adjusted to pH 7.6. A control with 0.5 ml. of water was also set up in each case. All were incubated at 38° for 30 minutes and the remaining activities estimated by adding the requisite quantities of magnesium and pyrophosphate as in the standard assay, the volume of buffer being adjusted to make the final volume to 5 ml. Different enzymes (E_1, E_2, E_3) are used. Assay period, 15 minutes; temperature, 38°; pH 7.6.

Inhibitor	Per cent activity remaining after treatment								
	Water-treated enzyme			Pyrophosphate-treated enzyme			Magnesium-treated enzyme		
	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3
Water	74	61	94	101	83	100	100	79	101
0.1 M formaldehyde			<10			48			<10
0.1 " formate	49	46		97	81		98	79	
0.1 " acetate	41	35		98	87		96	85	
0.1 " chloroacetate	37	31		100	93		99	89	
0.1 " iodoacetate	6	0	21	35	28	69	17	13	52
0.1 " malonate	17	10	25	100	98	98	88	81	90
0.1 " oxalate			<10			92			71
0.1 " succinate			54			104			102
0.1 " aspartate			51			100			98
0.1 " malate			33			98			99
0.1 " tartrate			53			102			101
0.1 " fumarate			15			96			97
0.1 " maleate			26			99			98
0.1 " citrate			<10			97			96
0.1 " guanidine			18			34			23
0.1 " thiourea			26			100			91
0.01 M ascorbate			37			100			93
0.01 " dehydroascorbate*			21			95			78
0.01 M alloxan			64			92			68
0.001 M copper sulfate			<10			<10			<10
0.001 " sodium fluoride			<10			19			14

* Dehydroascorbic acid is prepared by treating ascorbic acid solution with norit.

Reversal Experiments

A few inhibitors were selected to ascertain the extent the activators cysteine and bile salt could reverse the reactions. The enzyme is incubated with the inhibitors for 15 minutes and then with cysteine or bile salt for another 15 minutes and the activities are measured. For purposes of comparison, incubations were carried out at room temperature (28°) and at 38°. The results are given in Table VII.

TABLE VII

Reversal of Inhibition by Cysteine and Bile Salt

0.5 ml. of the enzyme was incubated with 0.5 ml. of the reagents for 15 minutes, and the activities were then measured, as shown in Column A. Two sets of similarly treated and incubated enzymes were treated with 0.5 ml. of 0.5 per cent sodium taurocholate (Column B) or 0.5 ml. of 0.1 M cysteine (Column C), and incubated for another 15 minutes and the activity measured. The preliminary incubation of Enzyme E$_1$ was carried out at room temperature, and of Enzyme E$_2$ at 38°. Enzymes E$_3$ and E$_4$ were incubated with the inhibitors at 38° and with the activators at 28°. All of the reagents were adjusted to pH 7.6. Assay period 15 minutes; temperature 38°; pH 7.6.

Reagent added	Per cent activity		
Enzyme E$_1$			
	(A), 28°	(B), 28°	(C), 28°
Water	93	130	135
0.001 M copper sulfate	<5	<5	87
0.001 M sodium fluoride	<5	11	17
0.1 M formaldehyde	<10	<5	<10
0.1 “ iodoacetate	27	26	31
0.1 “ acetate	59	103	78
0.01 M alloxan	64	97	109
0.04 “ veronal sodium	61		99
Enzyme E$_3$			
	(A), 38°	(B), 28°	
Water	100	105	
0.1 M acetate	70	90	
0.05 M veronal sodium	41	40	
0.05 “ alloxan	<5	<5	
0.05 “ oxalate	<5	<5	
0.05 “ malonate	62	76	
0.05 “ citrate	<5	<5	
0.1 M fumarate	26	54	
0.1 “ maleate	54	72	

Reagent added	Per cent activity		
Enzyme E$_2$			
	(A), 38°	(B), 38°	(C), 38°
Water	97	92	88
0.1 M oxalate	<5	<5	60
0.1 “ malonate	12	22	37
0.1 “ citrate	<5	<5	55
0.1 “ maleate	47	65	37
0.1 “ thiourea	18	15	75
0.1 “ guanidine	<5	<5	60
5 mg. ascorbic acid	18	20	84
Enzyme E$_4$			
	(A), 38°		(C), 28°
Water	100		105
0.1 M acetate	76		85
0.05 M veronal sodium	41		61
0.05 “ alloxan	<5		52
0.05 “ oxalate	19		59
0.1 “ malonate	24		43
0.1 “ fumarate	31		27
0.1 “ maleate	61		53

DISCUSSION

It is difficult to understand the mechanism by which fluoride inhibition is brought about, because pyrophosphate and magnesium could not protect the enzyme nor could the bile salt and cysteine reverse the fluoride inhibition. Copper inactivation must be due to its action on the enzyme thiols since cysteine reverses the inhibition. The negligible protection by Mg and partial protection by pyrophosphate against formaldehyde, whose inhibition is irreversible either with bile salt or with cysteine, suggest that the inhibition must have been brought about by interaction with the amino groups of the enzyme, and that Mg protects only the SH groups and not the amino groups, whereas pyrophosphate could protect the amino groups also. More or less similar results are obtained with iodoacetate. *Does iodoacetate produce inhibition by reacting also with amino groups?* Such a possibility was first suggested by Michaelis and Schubert (27) and later by others (28, 29). The results of Williams and Watson (19) and Bodansky (31) indicate the possibility of amino groups inhibiting alkaline phosphatases, which could be reversed by iodoacetate. Though it is generally believed that iodoacetate is a specific thiol inhibitor (30), it might not be correct to regard all enzymes that are inactivated by iodoacetate as thiol enzymes, or vice versa (32). Hence it is more likely that iodoacetate inhibition of the enzyme is brought about primarily by its action on amino groups.

Alloxan inhibition could be reversed partly with bile salt and completely with cysteine, provided the concentration of the alloxan was low and the incubations were performed at room temperature. Higher concentrations and incubation at 38° produced inhibitions which could not be reversed with bile salt and could only be partly reversed with cysteine. Acetate inhibition is reversed to a greater extent with bile salt than with cysteine, indicating some kind of blocking or shielding of the thiols by acetate.

Veronal inhibition could not be reversed with bile salt, but reversal occurs to a certain extent with cysteine. Malonate inhibition could be reversed only to a small extent either with cysteine or with bile salt. Oxalate and citrate are more toxic than malonate, the inhibitions being irreversible with bile salt. But their inhibitions, as well as those due to entirely different compounds like thiourea, guanidine, and ascorbic acid, could be reversed to a large extent with cysteine.

Results with fumarate and maleate offer additional difficulties in correlating the above findings. Morgan and Friedmann (33) claim that maleic acid is a specific thiol inhibitor, whereas fumaric acid has no action on thiol enzymes. Further, different thiol enzymes are inhibited to a varying extent with maleic acid (34). In the present study it was found that fumarate is more toxic than maleate and that cysteine could not reverse their inhibitions. The fumarate inhibition has only to be explained as due to shielding the active centers by the trans isomer, the steric effects due to the cis isomer being only slight. Reversal with bile salt supports such an assumption. How far maleate reacts with the thiols producing some fumarate simultaneously and how far this rearranged isomer contributes to the inhibition it is difficult to assess, but such probabilities exist. In passing it will be interesting to note the findings of Greenberg and Winnick (35).

The results with guanidine and thiourea are interesting and need to be studied more extensively. Both seem to react with the thiols, since cysteine reverses their inhibitions. But they differ from each other in that the pyrophosphate and Mg could protect the enzyme only against thiourea and not against guanidine. Work on the effect of these two reagents on other thiol enzymes might perhaps throw some light on the nature of their action.

Besides the thiol groups indicated by the copper and alloxan inhibitions and reversal with cysteine, and the amino groups indicated by the irreversible inhibitions with formaldehyde and iodoacetic acid, there is no indication of any probable presence of a metallic group as an active group. Though diethyl dithiocarbamate produced inhibition in higher concentrations, the reagent in smaller concentration as well as sodium azide did not cause inhibition, but, on the other hand, sometimes produced slight activation owing probably to the removal of trace metals present as impurities.

Until precise quantitative data on the kinetics of the inhibitions detailed in this paper are available, any conclusions drawn are only tentative. Since obtaining the enzyme in a purer form is prerequisite, work is being carried out in this direction.

SUMMARY

1. The rapid inactivation of erythrocyte pyrophosphatase on incubation with veronal-acetate buffer was found to be due to the inhibiting action of both the buffer constituents, *viz.* veronal and acetate.

2. Several monocarboxylic and dicarboxylic acids were studied and it was found that all inhibit the enzyme. The inhibitory action of the carboxyl group of the acetate is increased by substituting another carboxyl group in the acetate as in malonate, whereas introduction of an amino group as in glycine abolishes the original inhibitory action of the carboxyl. The intact carboxyl group seems to exert an inhibitory effect, since acetamide is practically non-inhibitory.

3. Though more carboxyl groups increase the inhibitor effect, the spatial configuration of the molecule decides the extent of inhibition. The proximity of the carboxyls seems to enhance the inhibitor effect. Oxalate is a very strong inhibitor, malonate is less inhibitory, and succinate is not as inhibitory as acetate. However, fumarate and maleate are more inhibitory than succinate, and curiously enough the trans isomer is as strongly inhibitory as malonate, while maleate is much less inhibitory.

4. Urea is non-inhibitory, whereas thiourea and guanidine are strongly inhibitory.

5. Both ascorbic and dehydroascorbic acids are inhibitory.

6. Among the amino acids tested, glycine, alanine, and phenylalanine are non-inhibitory, whereas tyrosine, tryptophan, and cystine are inhibitory.

7. Both pyrophosphate and magnesium ions are found to protect the enzyme to a large extent against many of the inhibitors. Against copper and fluoride they failed to protect the enzyme. Magnesium could not protect the enzyme against formaldehyde inhibition, while 50 per cent protection is obtained with pyrophosphate. Both showed poor protection against guanidine. Pyrophosphate had relatively more effect than magnesium against oxalate, iodoacetate, alloxan, and dehydroascorbic acid.

8. Besides sodium taurocholate, cysteine and glutathione are found to be activators of the enzyme. Sodium hyposulfite and hydrogen sulfide also produced some activation, but in some experiments they caused inhibition also. Cysteine is found to be the most potent activator, while cystine is an inhibitor.

9. Fe alone is very slightly activating, but addition of Fe neither reverses vitamin C inhibition, as in the case of papain (24, 25), nor does it increase cysteine activation, as in the case of liver pyrophosphatase (21).

10. Inactivation due to copper, alloxan, dicarboxylates except maleate and fumarate, ascorbate, veronal, acetate, guanidine, and thiourea could be reversed to a variable extent with cysteine. Bile salt could reverse to a small extent the inhibition due to fumarate and maleate, malonate and acetate. Neither cysteine nor bile salt could reverse the inhibition due to formaldehyde and iodoacetate. Fluoride inhibition could be reversed only to a negligible extent by both.

11. The various aspects of the inhibition, protection, activation, and reversal of the inhibitions have been discussed, and it is suggested that free sulfhydryl and the amino groups are essential for the enzyme action.

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UTILIZATION OF OPTICAL ISOMERS OF METHIONINE AND FORMYLMETHIONINE BY SOME LACTOBACILLI

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Reports on the utilization of the optical isomers of methionine by lactobacilli are conflicting. Stokes and Gunness (1) reported that *Lactobacillus arabinosus* does not utilize D-methionine. Dunn *et al.* (2) reported that D-methionine was inactive in producing growth of *L. arabinosus* and *Leuconostoc mesenteroides*, and Lyman *et al.* (3) made a similar observation with *L. mesenteroides* and *Streptococcus faecalis* R. However, Henderson and Snell (4) stated that D-methionine has partial activity with *S. faecalis* R in the presence of L-methionine, and Steele *et al.* (5) claimed that D-methionine was 10 per cent as active as the L isomer with *L. mesenteroides*.

The object of this investigation was to determine the relative degrees of utilization of pure optical isomers of methionine, formylmethionine, and racemic DL-methionine by *L. arabinosus*, *L. mesenteroides*, and *S. faecalis* R.

EXPERIMENTAL

DL-Methionine was resolved by a previously described (6) modification of the methods of Windus and Marvel (7) and Jackson and Block (8), in which purity of the isomers rather than high yields was stressed. Analytical data and optical rotations showing the purity of the isomers obtained by resolution of two 30 gm. quantities of formyl-DL-methionine are recorded elsewhere (6). The DL-methionine used for comparison with L-methionine was obtained by recrystallization of 2 gm. of analytically pure DL-methionine from 75 ml. of 67 per cent ethanol. The product weighed 1.4 gm. and contained 9.45 per cent nitrogen; theoretical, 9.39 per cent.

Cultures of *L. arabinosus* 17-5, *L. mesenteroides* P-60, and *S. faecalis* R were obtained from the American Type Culture Collection, 2029 M Street, Washington, D. C.

Microbiological analyses were made with the medium of Henderson and Snell (4) except that pyridoxine was used instead of pyridoxal; the volume of medium used was 1 ml. and that of the sample was 1 ml. Organisms were allowed to grow for 66 to 72 hours in the dark at 30°. The samples were then diluted to 50 ml., and the lactic acid was titrated with 0.05 N sodium hydroxide, brom thymol blue being used as indicator. Conventional standard curves were obtained from the average results of duplicate or quadruplicate tests over the concentration range of 2, 4, 6, 8, and 10 γ of L-methionine (Run II (6)) per test.

RESULTS AND DISCUSSION

It is apparent that D-methionine in a DL mixture is not utilized by *L. arabinosus* and *L. mesenteroides* because DL-methionine was only 49 ± 1 per cent and 51 ± 1 per cent as active with these organisms, respectively, as was L-methionine. These results were determined from a standard curve made by averaging the values of quadruplicate tests at concentration levels of 0, 2, 4, 6, 8, and 10 γ of L-methionine and from the average of quadruplicate tests containing 4, 8, and 12 γ of DL-methionine. D-Methionine in a DL-mixture appeared to have partial activity with *S. faecalis* R as noted by Henderson and Snell (4), but the amount of activity was not determined because results of various tests were erratic.

TABLE I

Growth Responses of Lactobacilli to D-Methionine and Formyl-D- and L-Methionine

Substance	Quantity used per test	Amount of Growth*		
		L. arabinosus	*L. mesenteroides*	*S. faecalis* R
	γ	*per cent*	*per cent*	*per cent*
D-Methionine	12	3.5	1.8	0.0
	20	3.8	2.4	0.0
Formyl-D-methionine	12	2.2	0.0	0.7
	20	2.8	0.0	1.4
Formyl-L-methionine	12	3.1	2.2	†
	20	4.7	3.1	†

* Approximately maximum growth of *S. faecalis* R, *L. mesenteroides*, and *L. arabinosus* was obtained with 12, 12, and 16 γ of L-methionine, respectively. The amount of growth produced by each substance was estimated by determining the apparent weight of L-methionine in the given weight of each substance tested, multiplying by 100, and dividing by the above approximate quantities of L-methionine, which gave maximum growth with respective organisms.

† Maximum growth.

The activities of D-methionine alone and of formyl-D- and L-methionine are shown in Table I. None of the organisms was capable of utilizing appreciable amounts of D-methionine or formyl-D-methionine when 12 and 20 γ of each were used per test. Formyl-L-methionine was not utilized by *L. arabinosus* or *L. mesenteroides*, but it was utilized by *S. faecalis* R as well or slightly better than was free L-methionine. The growth of *S.*

faecalis R was 12 ± 4 per cent greater with the formyl derivative than with L-methionine as determined by the average of quadruplicate tests with quantities of formyl-L-methionine equivalent to 2, 4, and 8 γ of L-methionine. Simmonds and Fruton (9–11) recently reported that a *prolineless* mutant of *Escherichia coli* grew better in a medium containing L-proline peptides than in one containing equimolar quantities of proline. A possibly similar relationship of utilization mechanisms between *S. faecalis* R and formyl-L-methionine and L-methionine is recognized.

These observations are pertinent to the concept that microorganisms require free amino acids for growth. Thus *S. faecalis* R is not able to utilize benzoyl-DL-tryptophan as shown by Stokes *et al.* (12) or acetyl-L-tryptophan (13). Although it is not known whether this organism can utilize formyl-L-tryptophan, it is apparent that utilization of *N*-acyl amino acids by lactobacilli is not determined generally by the nature of the acyl group because *L. arabinosus* and *L. mesenteroides* cannot utilize formyl-L-methionine. This observation indicates need for further investigation to determine the fundamental relationship of amino acid derivatives to their utilization by various microorganisms.

SUMMARY

Lactobacillus arabinosus and *Leuconostoc mesenteroides* do not utilize D-methionine alone or in the presence of L-methionine in DL-mixtures. *Streptococcus faecalis* R does not utilize D-methionine alone, but in the presence of L-methionine it appears to make partial use of the D form. None of these organisms utilized formyl-D-methionine. *L. arabinosus* and *L. mesenteroides* did not utilize formyl-L-methionine, but *S. faecalis* R gave 12 per cent more growth with this derivative than with free L-methionine. Need for further study of the relationship of amino acid derivatives to their utilization by microorganisms is pointed out.

Addendum—Since this paper was submitted for publication, Camien and Dunn (14) have shown that *L. arabinosus* 17-5 utilizes D-methionine and DL-methionine as well as does L-methionine in a synthetic medium containing at least 1 γ per cent of pyridoxamine or 10 γ per cent of pyridoxal. Pyridoxine is, however, ineffective in promoting utilization of D-methionine with this organism. The ineffectiveness of pyridoxine in promoting utilization of D-methionine by *L. arabinosus* is confirmed by the present work.

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MAGNESIUM VINYL PHEOPORPHYRIN a_5, ANOTHER INTERMEDIATE IN THE BIOLOGICAL SYNTHESIS OF CHLOROPHYLL

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Among the mutants arising after irradiation of *Chlorella vulgaris* with x-rays, three yellow mutants have been found, the absorption spectrum of whose ether extracts contained bands at 623 and 572 mμ. These bands were suggestive of protochlorophyll or a related compound. However, the concentration of this pigment was so low that its isolation in a crystalline state was not feasible. The pale green compound has therefore been characterized principally by the solubility properties and quantitative absorption curves of a number of its derivatives. On the basis of these properties the compound has been identified as Mg vinyl pheoporphyrin a_5, *i.e.* protochlorophyll, lacking phytol (Fig. 10).

The molar extinction coefficients of a number of the pertinent compounds, measured in dioxane, are presented in Table I, where E mole = $\log_{10} (I_0/I) \times (1/(\text{cm.} \times \text{mole per liter}))$.

EXPERIMENTAL

General Characteristics of Mutant—One of these mutants, *i.e.* strain 31, contained somewhat more of the pale green pigment than did the two others and has been used for identification of the pigment. The three mutants have similar characteristics. When grown in the dark on a solid medium of agar, glucose, and inorganic salts (1), they form deep yellow colonies tinged faintly greenish. They were found to contain carotenoids and a trace of chlorophyll besides the two-banded pale green pigment. When grown in the light, the cells become deep green and the two bands at 623 and 572 mμ are no longer detectable. In the light these mutants behave like wild type *Chlorella*, growing in the absence of glucose, *i.e.* photosynthesizing; when such green cultures are transferred to the dark, they maintain their green color for weeks, as does the wild type. Inocula taken from mutants grown in the light and seeded on a glucose medium give rise again in the dark to deep yellow colonies. In wild type *Chlorella* enzymes are present which bring about the production of chlorophyll in the dark as well as in the light. In contrast to the wild type, this yellow *Chlorella* mutant resembles the higher plants in requiring light for the production of chlorophyll.

Growth on solid medium containing glucose was best at 20–25°, a maximum yield being obtained in 7 to 9 days. For example the relative yield of packed cells at 20–25° was 1.3 cc.; at 15° it was 0.5 cc.; at 28–34° it was 1.1 cc.; and in the light on the same medium it was 1.5 cc. The yield of the pale green pigment was not appreciably affected by changing the conditions of growth or by enrichment of the medium with various substrates and vitamins.

TABLE I

Molar Extinction in Dioxane × 10^{-4}

Wave-lengths in mμ (upper figures of the pairs). The extinction (lower set of figures) was read with the Beckman spectrophotometer.

	Maximum I	Maximum II	Maximum III	Maximum IV	Soret maximum
Position of maxima					
Vinyl pheoporphyrin a_5 monomethyl ester	638 0.215	588 1.40	567 1.770	525 0.825	419 19.3
Pheoporphyrin a_5 monomethyl ester	634 0.188	583 1.261	562 1.606	521 0.939	417 19.3
Pheoporphyrin a_5 dimethyl ester oxime	625 0.194	573 1.074	550 1.440	512 1.290	
Chloroporphyrin e_6 trimethyl ester	629 0.170	576 0.673	543 0.809	506 1.263	
Vinyl chloroporphyrin e_6 trimethyl ester	633 0.130	579 0.720	550 1.04	512 1.14	409 20.5
Position of minima					
Vinyl pheoporphyrin a_5 monomethyl ester	623 0.110	575 1.15	543 0.490	475 0.180	
Pheoporphyrin a_5 momethyl ester	619.5 0.087	571.5 0.928	541 0.516		
Pheoporphyrin a_5 dimethyl ester oxime	610 0.077	562 0.520	533 0.470		
Vinyl chloroporphyrin e_6 trimethyl ester	620 0.085	565 0.480	530 0.410	465 0.201	

For the study of the pigment the cells were grown in the dark in Blake flasks. Each flask containing 700 cc. of medium (agar-glucose-inorganic salts) was inoculated with cells obtained from one agar test-tube slant. After 6 to 7 days at room temperature the cells were harvested by washing with about 20 cc. of water per flask and then centrifuging. The supernatant was tinged pale green and overlay a thin layer of young pale green cells and cellulose wall débris. The main bulk of the cells below this layer was deep yellow in color, consisting of large cells filled with starch granules. The total volume of packed cells per flask was about 5.0 cc.

Pigments of Chlorella Mutant—The harvest of one flask was treated with 40 cc. of acetone and after 1 hour was centrifuged. The residue was then extracted with 40 cc. of methanol and finally with 20 cc. of ether. The extracts were combined, diluted with water, and the ether layer was washed with water. A few cc. of saturated aqueous NaCl aided in the separation of the layers. The washed ether solution was diluted to 50 cc. with ether and the absorption spectrum of the total pigment extract was measured with a Beckman spectrophotometer in cells of 1 cm. light path (Curve A, Fig. 1).

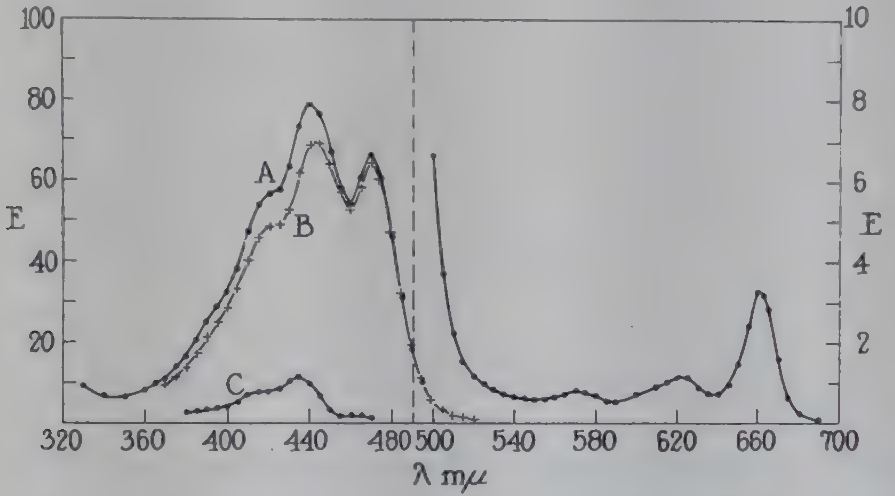

FIG. 1. Relative extinction in ether of pigments of *Chlorella* mutant 31 per flask of medium. Curve A represents extinction of total pigments. Curve B is the total absorption of the carotenoids. Curve C, obtained by difference, represents the extinction of the green pigments in the ultraviolet; the extinction of the green pigments in the visible region is represented by Curve A in the visible region.

To determine the absorption of the green pigments in the ultraviolet region it is necessary to remove the carotenoid pigments. The green pigments are made water-soluble with methyl alcoholic KOH and the absorption of the yellow pigments is measured (Curve B); the difference between the extinction values of Curve B and Curve A is plotted in Curve C, which is the absorption spectrum of the green pigments in the ultraviolet region. The separation of the green and the yellow pigments was brought about by adding to a small separatory funnel 2.5 cc. of the ether extract plus 2.5 cc. of petroleum ether (b.p. 60–70°) plus 3 cc. of 30 per cent KOH in absolute methanol. The mixture was shaken intermittently for 5 minutes. Then 3 cc. of water and 3 cc. of ether were added. The upper yellow ether layer was removed. A trace of yellow pigment remained in the lower layer, to which was added 1 cc. of methanol; the solution was then extracted with 5 cc. of ether. The yellow ether extracts were combined, washed with water, and diluted to 25 cc. This volume corresponds to a 1:10 dilution of the original stock ether solution. No fluorescence with ultraviolet light was detectable in this yellow ether solution, indicating the absence of porphyrin or dihydroporphyrin pigments. The absorption of this extract is presented in Curve B of Fig. 1.

The band maxima at 620 and 430 mμ are interpreted as representing Mg vinyl pheoporphyrin a_5, and the maximum at 419 as that of vinyl pheoporphyrin a_5. From the extinction maxima one may estimate that the 5 cc. of packed cells contained a total of about 0.16 mg. of chlorophyll *a* and approximately 2 mg. of yellow pigments.

Isolation and Absorption Spectrum of Pale Green Pigment—To obtain sufficient pigment the cells of mutant 31 were grown in batches of twenty to thirty Blake flasks in the dark on the agar-glucose-inorganic salt medium at 20–25°. They were harvested after 5 to 6 days, at a time when growth was not quite maximum, in order to diminish the yield of certain decomposition products. The cells and supernatant were stirred with 50 mg. of Na_2CO_3 and then treated with 4 volumes of acetone. After centrifugation the aqueous acetone extract was transferred to a separatory funnel and extracted three times with petroleum ether (b.p. 50–60°) to remove chlorophyll, carotenoids, and fatty substances. No absorption band at 625 mμ was present in the petroleum ether solution, indicating the absence of protochlorophyll. The acetone extract was diluted with water, acidified with M/15 KH_2PO_4, and extracted into ethyl ether twice and once again after addition of aqueous saturated NaCl. The resulting pale green ether solution was washed with water and then extracted with 0.05 M ammonia buffer at pH 9.5. It was then returned to ether and concentrated to a volume of 25 cc. The total yield of pigment in this extract was about 0.5 mg.

The absorption spectrum of the ether solution is shown in Fig. 2, Curve A. The position of the band maxima at 623, 570, 530, and 432 mμ corresponds to the position of the band maxima of protochlorophyll (2), but the ratios of the extinction maxima of the bands do not correspond to those of protochlorophyll. On analysis it was found that this absorption spectrum may be accounted for by assuming a mixture of Mg vinyl pheoporphyrin a_5 and vinyl pheoporphyrin a_5. Since the extinction values of Mg vinyl pheoporphyrin may be assumed when corrected by a weight factor to be identical with its phytyl ester (*i.e.* protochlorophyll), the extinction values of Koski and Smith (2) have been used, together with the extinction values of synthetic vinyl pheoporphyrin. From the extinction values at 623 and 419 mμ the experimental Curve A was found to be 1.2 × 10^{-5} M with respect to vinyl pheoporphyrin and 2.10 × 10^{-5} M with respect to the Mg complex. By subtracting the extinction values of vinyl pheoporphyrin from Curve A, the points on Curve B were obtained. Curve B should correspond to the absorption spectrum of pure Mg vinyl pheoporphyrin a_5. For protochlorophyll minus its phytol the ratios of E (the molar extinction coefficient) for the band maxima at 623, 571, and 432 mμ are 1.0:0.427:9.30. For Curve B the ratios are 1.0:0.440:9.07. The agreement is satisfactory and indicates that the absorption of the pale green extract from *Chlorella* was due primarily to vinyl pheoporphyrin and its Mg complex.

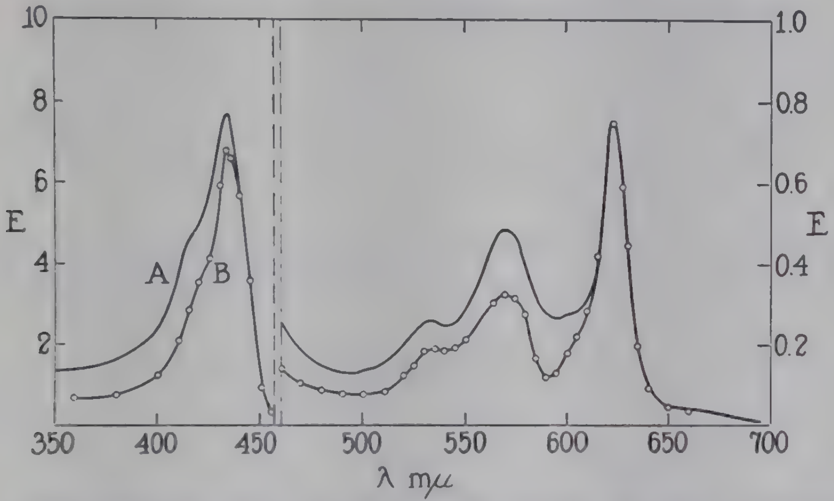

FIG. 2. Relative extinction of the pale green pigment isolated by extraction, indicating that the solution contains a mixture of vinyl pheoporphyrin a_5 and its Mg complex. Curve A represents total pigment. Curve B represents the magnesium complex. The points on Curve B were obtained by subtraction of extinction values of vinyl pheoporphyrin; the ratios of the maxima of Curve B agree with published data on the Mg complex.

Quantitative Determination of Mg^{++} in Complex—On shaking some of the pale green ether solution with 1 N HCl and washing out the acid, the ether solution was found to contain a porphyrin spectrum of the rhodo type (3). Evidently a metal atom was split out of the complex. Conditions were chosen to remove the metal, estimate it quantitatively, and at the same time recover the porphyrin for further study.

The pale green ether solution was washed with M/15 $NH_4H_2PO_4$ and then with water. The extinction value for this solution at 623 mμ was determined. Assuming it to be Mg vinyl pheoporphyrin a_5, the solution was calculated to be 4.5 × 10^{-6} M. Of this solution 170 cc. were concentrated to a volume of a few cc. and transferred with ether washings to a special graduated centrifuge tube whose lower 5 cm. portion had a capacity of 1 cc. and whose upper portion was flared out. To the deep green ether solution of 3.5 cc. was added 0.1 cc. of 3 N HCl, and the acid was stirred into the ether by bubbling air through it with a capillary pipette. The acid was then neutralized exactly with 0.1 cc. of 3 N NaOH. The ether was removed by evaporation. Water was added to dilute the solution in the tube to 0.8 cc. and the tube was centrifuged to spin down the insoluble porphyrin. The resulting solution had a very faintly yellowish tinge, owing to a trace of colloidal porphyrin. Of this solution 0.1 cc. was used for the Mg determination by the Titan yellow method (see below). To correct for the absorption due to the colloidality, 0.1 cc. of the metal-containing solution was diluted to 1 cc. with the reagents except Titan yellow and its extinction was determined against a blank of the reagents without Titan yellow. The resulting extinction was subtracted from the colorimetric Mg reading. On the assumption of Mg vinyl pheoporphyrin and the extinction at 623 mμ, the total ether sample used was calculated to contain 18.6 γ of Mg^{++}; found by the colorimetric procedure, 19.2 γ of Mg^{++}.

Spectrophotometric Comparison of Porphyrin with Vinyl Pheoporphyrin a_5—The quantitative absorption spectrum of vinyl pheoporphyrin a_5 has not been reported in the literature. We prepared this compound from dried spinach leaves by way of chlorophyll *a* + *b* precipitation in petroleum ether (4), hydrolysis of the phytol with strong acid (5), separation and crystallization of pheophorbide *a* from ether (5), reduction of the dihydroporphyrin with iron and formic acid, its reoxidation by air to vinyl pheoporphyrin a_5 monomethyl ester, and its crystallization from ether (6). Calculated for $C_{35}H_{34}O_5N_4$, C 71.2, H 5.76 per cent; found, C 70.42, H 6.15, and no ash.

The molar extinction in dioxane of this synthetic vinyl pheoporphyrin a_5 monomethyl ester (Fig. 3, I) is presented as the smooth curve, B, in Fig. 4. (Because of the great tendency toward peroxide formation of dioxane, this solvent was distilled over sodium and stored in small bottles filled to the brim, tightly stoppered, in the ice box.) The extinctions are identical for dioxane and ether as solvents, except that in ether the maxima are displaced about 1 mμ to the ultraviolet side.

The ether solution containing the pale green compound isolated from *Chlorella* was treated with 0.1 N HCl to split out the Mg^{++}. The ether solution was then washed with water and extracted successively with increasing concentrations of HCl. The main component, extractable between 2.7 and 3.3 N HCl (HCl No. $\simeq$ 11), when transferred into ether had a four-banded rhodo type spectrum in the visible region whose most intense band was at 566 mμ.

Small amounts of other rhodo type substances were observed. One having an HCl No. $\simeq$ 10 had its most intense band at 562 mμ; another

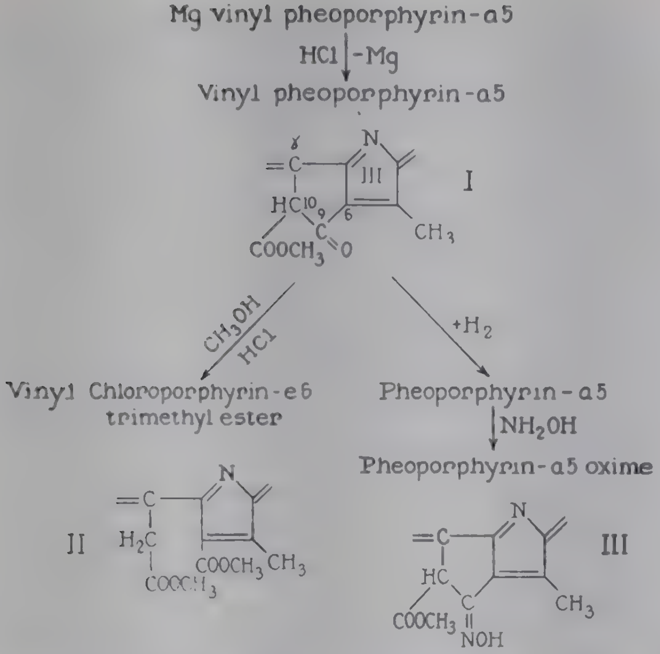
Mg vinyl pheoporphyrin-a5
HCl
-Mg
Vinyl pheoporphyrin-a5
N
γ
III
I
HC
10
9
6
C
CH3
COOCH3
O
CH3OH
HCl
+H2
Vinyl Chloroporphyrin-e6 trimethyl ester
Pheoporphyrin-a5
NH2OH
Pheoporphyrin-a5 oxime
II
H2C
COOCH3
COOCH3
CH3
III
HC
CH3
NOH

FIG. 3. Derivatives prepared from Mg vinyl pheoporphyrin a_5. Only the pyrrole Ring III and its adjacent atoms are shown.

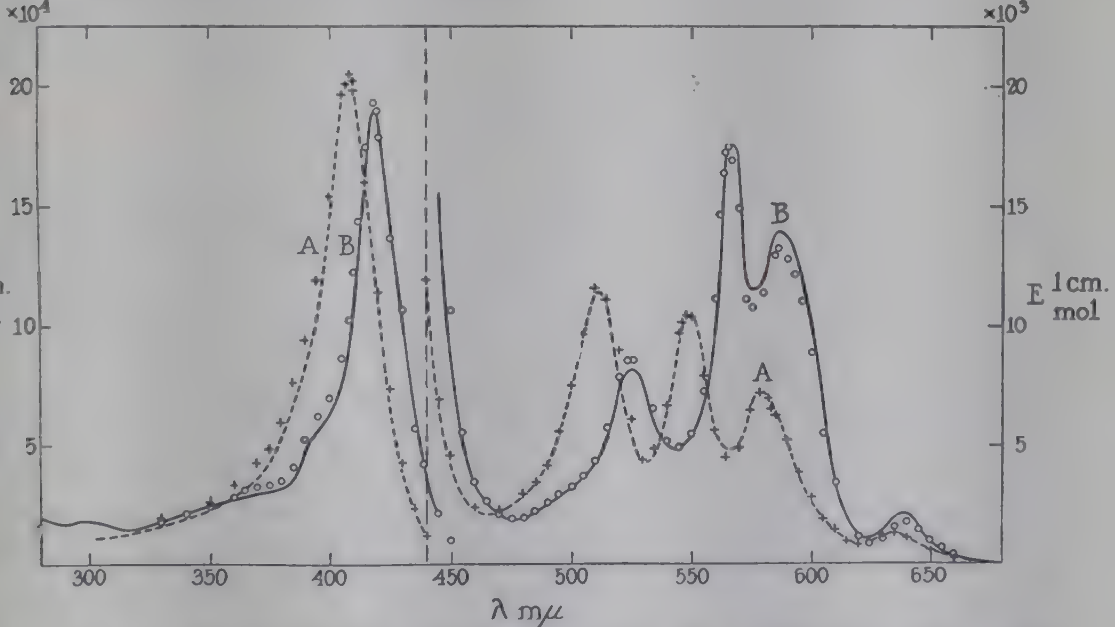

FIG. 4. Molar extinctions in dioxane of synthetic vinyl pheoporphyrin a_5 (Curve B). Synthetic vinyl chloroporphyrin e_6 (Curve A). The points on both curves represent extinction of compounds derived from the pale green pigment of *Chlorella* mutant 31.

having an HCl No. $\simeq$ 7 had its most intense band at 558 mμ. A trace of a chlorophorpyrin-like spectrum with an HCl No. $\simeq$ 0.4 was also observed once. These substances were noted to be increased especially when the cells were grown during the summer, the room temperature being

30–32°. The properties of these pigments suggested that they are decomposition products of vinyl pheoporphyrin, the main component.

To free the main component of the slight impurities the ether solution was extracted several times with 3.0 N HCl and the fraction was selected which was extractable between 3.0 and 3.3 N HCl. This acid solution was transferred to peroxide-free ether, the ether solution washed with water and evaporated, the residue dissolved in dioxane, and the absorption spectrum measured. The extinction at one point, *i.e.* 419 mμ, was multiplied by a factor to bring it to the molar extinction value of synthetic vinyl pheoporphyrin a_5 at this wave-length. All other points were multiplied by this same factor and are plotted as points on Curve B of Fig. 4. These points lie sufficiently close to the curve to suggest that the substance derived from the *Chlorella* mutant is vinyl pheoporphyrin a_5. The deviation of the points from the curve in the band regions at 525 and 587 mμ is beyond experimental error, indicating traces of impurity, possibly vinyl phylloerythrine or vinyl 10-hydroxypheoporphyrin or both.

The solubility properties of this compound derived from *Chlorella* are also in agreement with those of vinyl pheoporphyrin a_5 monoester in the value of the HCl number and in the extractibility in aqueous ammoniacal solution from ether, which indicates an ionizable carboxyl group. No evidence for a phytol ester was obtained.

The general characteristics of the absorption spectrum also support the idea that the derivative from *Chlorella* is a pheoporphyrin. The rhodo type spectrum (the third visible band being strongest) is produced by a formyl, acetyl, or carboxyl group attached to a β-carbon of a pyrrole in the porphyrin. The presence of a C═O group in pheoporphyrin is signalized by a rhodo type spectrum. However, as compared to rhodoporphyrin (in which a —COOH is attached to a β-carbon) the spectrum in pheoporphyrin is shifted some 17 mμ to longer wave-lengths owing to the C═O being part of a cyclopentanone ring. The displaced rhodo type spectrum is given by porphyrin compounds which possess a five- or six-membered ring containing a C═O group attached at the β-carbon atom.

Vinyl Chloroporphyrin e_6 Trimethyl Ester (Fig. 3, II)—A characteristic reaction of pheoporphyrin with intact isocyclic ring (*i.e.* 10-carbomethoxy-0-γ-ethanone) is methanolysis, resulting in the splitting of this ring and the formation of a trimethyl ester derivative. This derivative is readily recognized by its characteristic four-banded etio or step-ladder type of absorption spectrum in the visible region. Phylloerythrine (Fig. 5, VII) does not undergo this reaction. 10-Hydroxypheoporphyrin a_5 (IV) is converted to chloroporphyrin e_7 lactone (V), still of the rhodo type, and thence to pheoporphyrin a_7 trimethyl ester (VI) which possesses an etio type of spectrum.

The quantitative absorption spectrum of vinyl chloroporphyrin e_6 has not been reported in the literature. The trimethyl ester of this compound was prepared by adding to 6 mg. of crystalline synthetic vinyl pheoporphyrin a_5 15 cc. of a solution of dry methanol containing 30 per cent by weight of dry HCl. The solution was left overnight tightly stoppered at room temperature, then diluted with ice water, neutralized with sodium acetate, extracted into ether, and the ether solution fractionated with successively increasing concentrations of HCl. Two substances were present. The main component had an HCl No. ≃ 2.2, identifiable with the chloroporphyrin compound. It was crystallized from a mixture of chloroform and methanol. Its absorption spectrum in dioxane is represented as Curve A of Fig. 4. The other component, present in traces and of a rhodo type, had an HCl No. ≃ 13 to 14 and was presumably vinyl phylloerythrine methyl ester produced during the reaction.

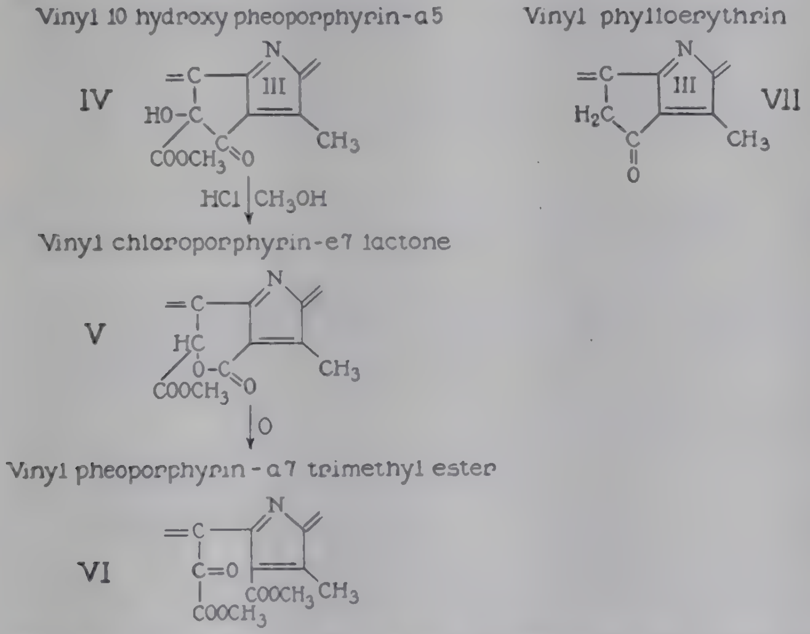
Vinyl 10 hydroxy pheoporphyrin-a5
IV
N
III
HO-C
COOCH3
O
CH3
HCl | CH3OH
Vinyl chloroporphyrin-e7 lactone
V
HC
O-C
COOCH3
O
CH3
O
Vinyl pheoporphyrin - a7 trimethyl ester
VI
C=O
COOCH3
COOCH3
CH3
Vinyl phylloerythrin
VII
H2C
C
O
CH3

FIG. 5. Other products arising from vinyl pheoporphyrin. Only pyrrole Ring III and its adjacent atoms are shown.

The pheoporphyrin compound derived from *Chlorella* was also treated in the same way with methyl alcoholic HCl. The main fraction with HCl No. ≃ 2.2 was isolated, neutralized, dissolved in dioxane, and its absorption spectrum measured, the data being plotted as points on Curve A of Fig. 4. The points are in essential agreement with the curve of the synthetic compound.

When *Chlorella* strain 31 was grown at 30–32°, compounds suggestive of decomposition of pheoporphyrin were observed. The ether solution extracted with 2.5 to 4 N HCl contained a mixture of compounds of the rhodo type. This mixture when treated with methyl alcoholic HCl and fractionated showed the presence of a relatively high content of a rhodo type compound with an HCl No. $\simeq$ 10 to 14, presumably vinyl phylloerythrine methyl ester. A fraction with HCl No. $\simeq$ 4 and the etio type of spectrum was presumably vinyl pheoporphyrin a_7 trimethyl ester, and a fraction with an HCl No. $\simeq$ 2.5 contained vinyl chloroporphyrin e_6 triester with a trace of the vinyl pheoporphyrin a_7 triester.

Identification of Pheoporphyrin from Chlorella Strain 31 As Monovinyl Compound—The hypothesis was presented in previous papers that protoporphyrin (containing two vinyl groups) is a precursor of chlorophyll *a* (containing one vinyl group). At some intermediate stage one of the vinyl groups is reduced to an ethyl group. Absorption spectrum data indicate that the wave-lengths of a porphyrin band system are decreased some 4 mμ on reduction of one vinyl group and about 8 mμ on reduction of two vinyl groups (3). With this criterion it could be demonstrated that the pheoporphyrin a_5 isolated from *Chlorella* was a monovinyl pheoporphyrin.

2 mg. of synthetic vinyl pheoporphyrin a_5 dissolved in dioxane were added to 2 cc. of acetic acid containing 2.5 mg. of colloidal Pd, heated on a boiling water bath, and H_2 passed into the solution for 10 minutes. The green solution was reduced to a yellow-brown color with an absorption band at 490 to 500 mμ. Water was then added and the solution transferred to a separatory funnel containing ether and shaken, and the compound permitted to autoxidize for 10 minutes. The fraction extractable between 2.5 and 3 N HCl was used. The resulting pheoporphyrin a_5 was dissolved in dioxane and its absorption determined. The extinction at 415 mμ was multiplied by a factor to convert it to the molar extinction value at this wave-length as determined by Stern and Wenderlein (7) and all the other points were multiplied by this factor. The maxima and minima of the resulting smooth curve (Fig. 6) are in good agreement with data reported by Stern and Wenderlein on pheoporphyrin a_5. The fraction isolated from the *Chlorella* mutant and extractable with 3.0 to 3.3 N HCl from ether solution was reduced in the way described above and isolated. Its absorption spectrum measured in dioxane, when converted by a factor to the coefficient of molar extinction, is represented as points on the smooth curve of Fig. 6. The wave-length maxima are decreased about 4 mμ, providing supporting evidence that the original compound from *Chlorella* contained only one vinyl group.

As a further check on the identity of the *Chlorella* compound, the compound reduced at the vinyl group was heated in aqueous pyridine solution with hydroxylamine and Na_2CO_3 for 30 minutes at 100° and transferred to ether. A fraction was isolated by extraction of the ether solution with 1.5 N HCl. The absorption spectrum of the compound in dioxane

(Fig. 7), when multiplied by a factor to convert it to the coefficient of molar extinction, was compared with the data of synthetic pheoporphyrin oxime dimethyl ester (Fig. 3, III) as published by Stern and Wenderlein

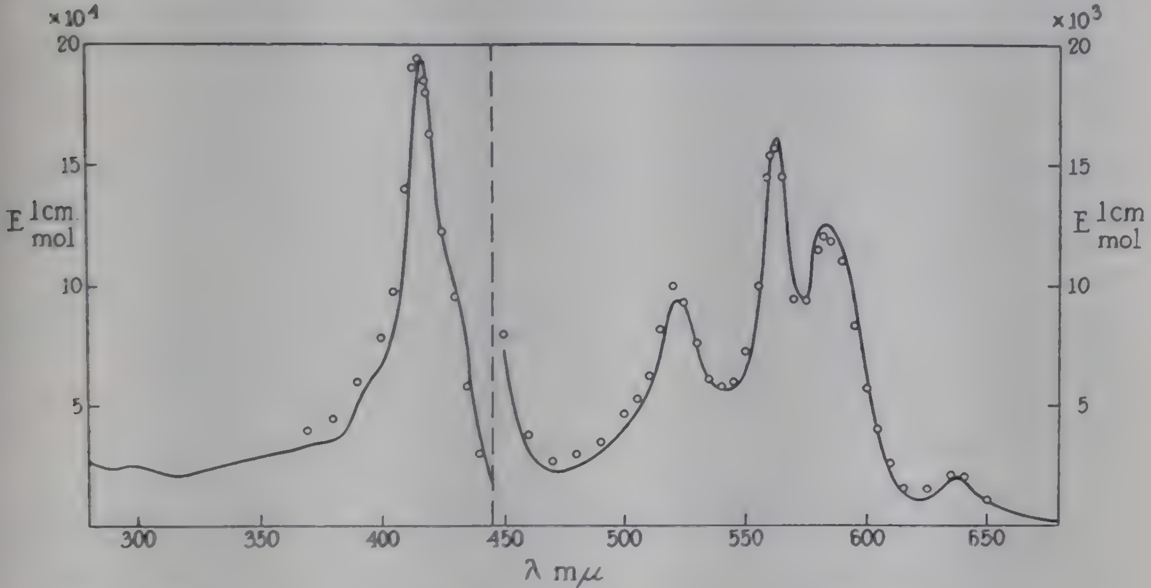

FIG. 6. Molar extinction of pheoporphyrin a_5 in dioxane. The smooth curve represents the synthetic preparation. The points on the curve represent the compound derived from *Chlorella*.

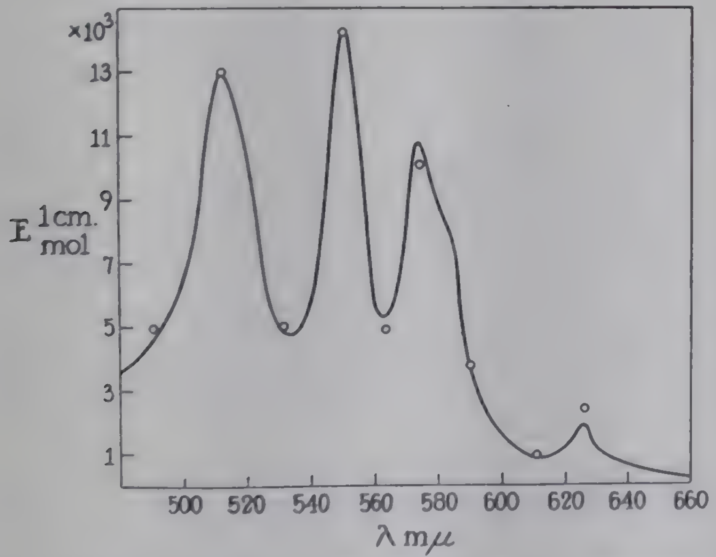

FIG. 7. Molar extinction of pheoporphyrin a_5 oxime in dioxane. The points on the curve are those of the compound derived from *Chlorella*.

(8). The points of maxima and minima fit their published curve and furnish additional support for the identification of the compound isolated from *Chlorella*.

Solubility Properties of Protochlorophyll from Etiolated Barley—The absence of a phytol group in the vinyl pheoporphyrin a_5 compound isolated from the *Chlorella* mutant led us to scrutinize the evidence that protochlorophyll was a phytol ester. Noack and Kiessling (9), in their important contributions to protochlorophyll structure, isolated "protochlorophyll" from cucurbit seed coats and also from etiolated oat seedlings. The properties of this compound, namely too low a C content of the impure preparation, extraction from ether with HCl of 12 to 18 per cent concentration, solubility properties that did not change on treatment with phytol esterase (chlorophyllase), all were indicative of the absence of phytol. These authors concluded that they had obtained no direct evidence for the presence of phytol. Although Fischer and Oestreicher (10) eventually synthesized the phytol ester of vinyl pheoporphyrin a_5, they did not determine the HCl number of their compound nor compare the solubility properties with protochlorophyll isolated from the plant.

The only direct evidence that protochlorophyll is a phytol ester is the Mg determination by Koski and Smith (2) of the protochlorophyll isolated from etiolated barley by chromatography, which agrees with the assumption of a phytol. Unfortunately no carbon analysis of this compound was made nor were significant solubility properties reported.

In order to obtain confirmatory evidence to support the presence of the phytol group we have studied the solubility of protochlorophyll from etiolated barley. In one experiment the etiolated seedlings were grown in six flats as described by Koski and Smith (2), harvested in the dark, and heated for 5 minutes in water at 90° to destroy the esterases. After the excess moisture was squeezed out, the material was ground with about 9 volumes of acetone in a blendor. The pigments were then extracted into petroleum ether (60–70°, b.p.) with the aid of saturated NaCl. Extraction of the petroleum ether solution with dilute aqueous 0.01 N NH_4OH removed about 10 to 20 per cent of the Mg pheoporphyrin compound, indicating that this was not esterified with phytol. To the main bulk of the Mg pheoporphyrin compound in petroleum ether were added 20 cc. of acetic acid and the petroleum ether was removed by evaporation at low pressure. The acetic acid solution was mixed with ethyl ether, and the ether solution was washed with water and was fractionated with increasingly higher concentrations of HCl in the cold room. A trace of vinyl pheoporphyrin a_5 was extracted with 4 N HCl, but the bulk of the compound was extractable with 8 N HCl; *i.e.*, at an acidity somewhat less than was required to extract the traces of pheophorbide *a* phytyl ester. The hydrolysis of the ester in strong acid is no greater in protochlorophyll than in chlorophyll. These solubility properties indicate that protochlorophyll as it exists in the etiolated barley contains an alcohol highly insoluble in water in ester linkage; *i.e.*, presumably a phytyl ester.

The absorption spectrum of this acid-extracted phytyl ester, transferred to ether, washed, evaporated, and dissolved in dioxane, was determined and multiplied by a factor to convert it to the coefficient of molar extinction. The points so obtained fall mainly on the smooth curve (Fig. 8) for synthetic vinyl pheoporphyrin a_5. A slight impurity is suggested by the band at 585 mμ and a trace of pheophorbide is made evident by the band at 660 mμ.

In another experiment etiolated barley seedlings were ground in acetone, the final concentration of acetone being 60 to 70 per cent. The protochlorophyll in the acetone extract, placed overnight in the ice box, lost its phytol. It became extractable from petroleum ether into a dilute alkaline aqueous solution, and was extractable from ether with 4 N HCl. The compound was crystallized from ether-methanol and its quantitative absorption spectrum in dioxane adjusted to the coefficient of molar extinction is plotted in Fig. 8.

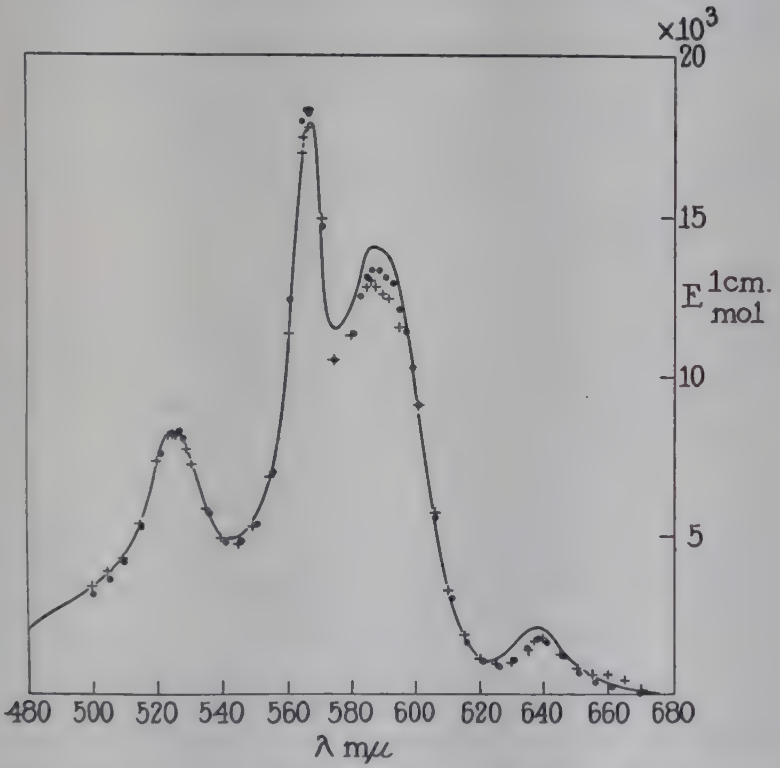

FIG. 8. Extinction of vinyl pheoporphyrin a_5 synthetic (smooth curve). ×, compound isolated from etiolated barley seedlings heated in boiling water and then extracted with ether and acid; ●, compound isolated from etiolated barley without heating and extracted with ether and acid.

These solubility properties may be considered as confirmatory evidence for the presence of a phytyl ester in the protochlorophyll molecule.

Some Experiments with Light—The lack of phytyl in the Mg vinyl pheoporphyrin compound obtained from the *Chlorella* mutant and the production of chlorophyll in this mutant in the light indicate complex relationships. To study chlorophyll formation three Blake flasks were seeded and grown in the dark for 7 days and then exposed to 150 foot-candles of fluorescent "daylight" at 6.5°. Quantitative extraction of the pigments was made as described above. The bands of Mg vinyl pheoporphyrin, originally present, disappeared and chlorophyll was formed in an amount equivalent to over twice this compound. At the same time there was a significant decrease in carotenoid absorption as compared to non-irradiated controls. In another experiment, flasks, similarly grown in the dark, were exposed to the light at a temperature of +1° to −2° for periods up to 12 hours. A progressive decrease in the Mg vinyl pheoporphyrin bands was observed with time without significant change in chlorophyll. A decrease in carotenoid absorption was also noted here.

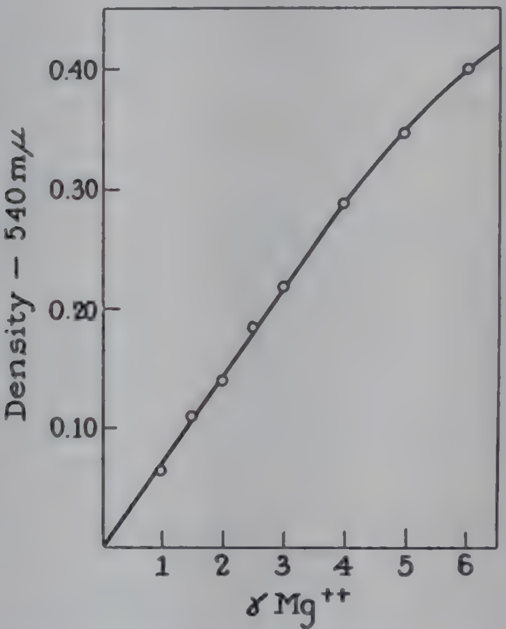

FIG. 9. Curve for microcolorimetric determination of Mg^{++}. The extinction values at 540 mμ of Mg in Titan yellow complex are plotted against Mg^{++} concentration.

Smith (11) demonstrated that protochlorophyll in etiolated barley was quantitatively converted to chlorophyll on illumination in the cold. The failure to observe chlorophyll formation at 0° in mutant 31 may be due to lack of phytol in the molecule, the Mg vinyl pheoporphyrin being destroyed on illumination. On illumination at 6.5° chlorophyll was synthesized, indicating that a chain of reactions including pheoporphyrin synthesis and esterification with phytol could occur at this temperature. Among other effects there seems to be an incomplete block to phytol esterification in this mutant.

Microcolorimetric Determination of Mg^{++}—The Titan yellow method of Ludwig and Johnson (12) was modified for the determination of microgram quantities of Mg^{++}. The colored solution with a total volume of 1 cc. is measured in a Beckman spectrophotometer at 540 mμ, with a narrow cell of 1 cc. capacity and a light path of 1 cm. (13). The extinction is measured against a blank containing all the reagents except Mg. The calibration curve for extinction against Mg concentration is a straight line up to about 4 γ of Mg (Fig. 9). The extinction values are stable for over an hour. The method is accurate to ±4 per cent. Accurate pipetting of the reagents, especially of the Titan yellow, is essential. The reagents are added in the following order: Mg^{++} (as $MgSO_4 \cdot 7H_2O$ for standard), 1 N H_2SO_4 0.02 cc., H_2O to make the solution up to 0.5 cc. The following are then added: 0.10 cc. of 1 per cent soluble starch (recently boiled), 0.20 cc. of saturated aqueous solution of $CaSO_4$, 0.10 cc. of 0.05 per cent aqueous solution of Titan yellow, 0.10 cc. of 1 N NaOH.

DISCUSSION

The properties of the pale green pigment, isolated from the *Chlorella* mutant 31 have been shown to agree in all respects with those of Mg vinyl pheoporphyrin a_5. The present status of the problem of biological chlorophyll synthesis may be represented by the scheme presented in Fig. 10.

By tracer techniques it has been shown that glycine and acetic acid or compounds derived from acetic acid are important building blocks in the formation of protoporphyrin (14). None of the intermediate steps to protoporphyrin is known. From *Chlorella* mutant W_5B protoporphyrin IX has been isolated. Magnesium porphyrin has been demonstrated in *Chlorella* mutant 60 (15). In this paper, evidence for the presence of Mg vinyl pheoporphyrin a_5 has been presented. For transformation to this stage from Mg protoporphyrin it is necessary to postulate several intermediate steps; one is the reduction of one of the vinyl side chains to an ethyl side chain; other steps are the oxidation of the propionic acid side chain, the esterification of this side chain with methyl alcohol, and the cyclization to form the cyclopentanone ring. The next step is the esterification of the second propionic acid group of Mg vinyl pheoporphyrin to form protochlorophyll. The last step, the reduction of protochlorophyll to chlorophyll, occurs in wild type *Chlorella* as an enzymic process in the dark. In *Chlorella* mutant 31 and in higher plants this process takes place in the light.

Two interesting relationships may be noted here. The two prominent pigments of protoplasm, namely heme and chlorophyll, are related structurally since they are derived from the same precursor, protoporphyrin IX.

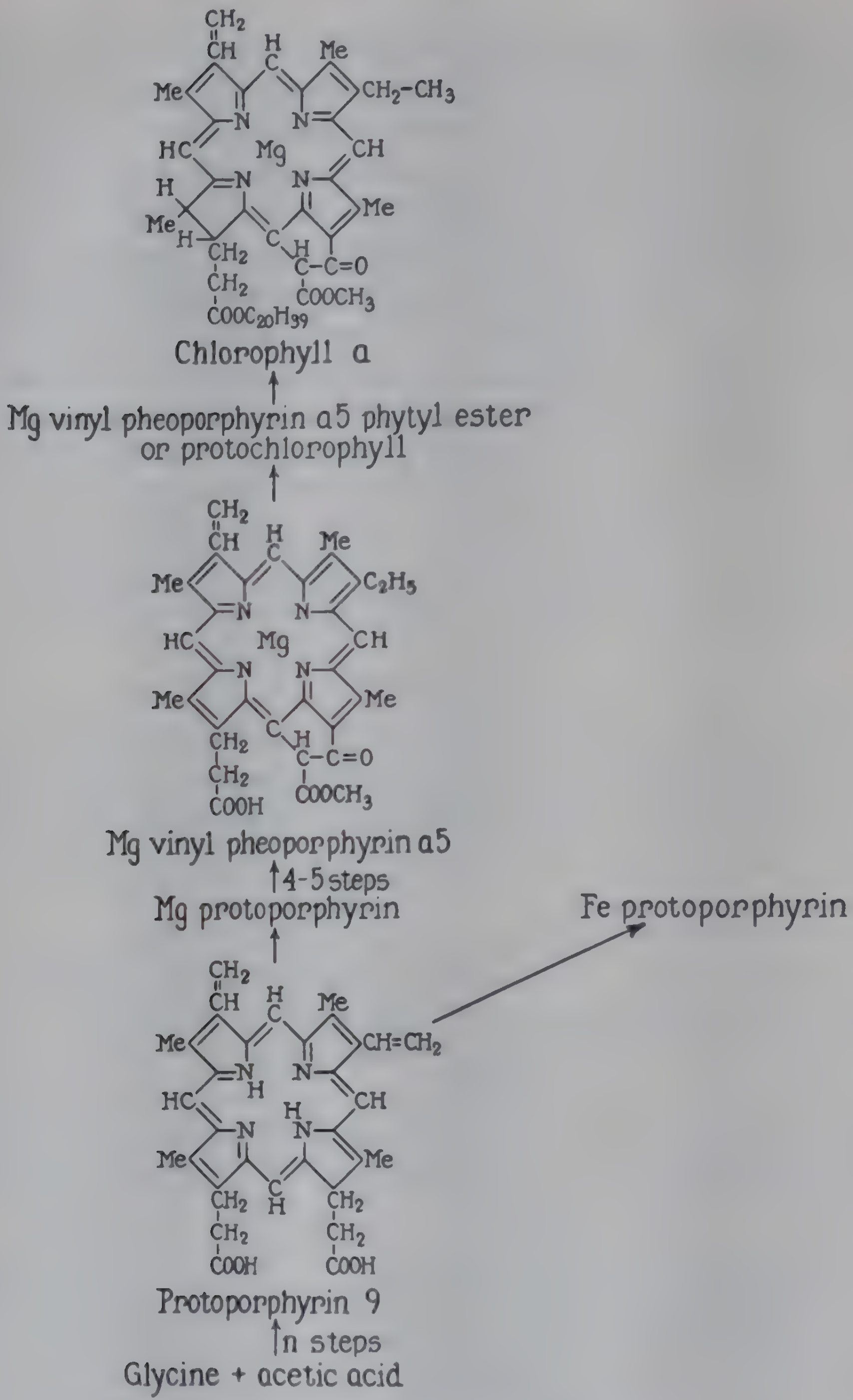
Chlorophyll a
Mg vinyl pheoporphyrin a5 phytyl ester
or protochlorophyll
Mg vinyl pheoporphyrin a5
4-5 steps
Mg protoporphyrin
Fe protoporphyrin
Protoporphyrin 9
n steps
Glycine + acetic acid

FIG. 10. Scheme of biosynthesis of chlorophyll

These two pigments are also related functionally as expressed in the following equation.

$$O_2 + 4H^+ + 4(e) \underset{\substack{\text{Chlorophyll} \\ nh\nu}}{\overset{\substack{-\Delta H \\ \text{Fe protoporphyrin}}}{\rightleftharpoons}} 2H_2O$$

In this equation (e) represents electrons of high potential energy contained primarily in the configuration of organic substrates; $-\Delta H$ is the heat liberated in oxidation; and $nh\nu$ is the number of light quanta stored. The forward reaction of oxidation with O_2 to form water takes place with a release of energy; this reaction is catalyzed by a series of heme enzymes which have Fe protoporphyrin as their prosthetic group. The reverse reaction, leading to a storage of energy, is the reaction of photosynthesis. Here the storage of the energy of sunlight is catalyzed by another porphyrin pigment, chlorophyll.

The significance of this scheme of heme and chlorophyll synthesis from the view-point of the evolution of oxidative and photosynthetic mechanisms is discussed elsewhere (16).

SUMMARY

From *Chlorella* mutant 31 a compound has been isolated with the properties of Mg vinyl pheoporphyrin a_5. Evidence supporting the identification of this compound is based primarily on spectrophotometric and solubility properties of a number of derivatives prepared from this compound.

Spectrophotometric analysis of the pale green pigment as isolated from *Chlorella* indicated that it was a mixture of vinyl pheoporphyrin a_5 and its Mg complex. On the assumption of Mg vinyl pheoporphyrin a_5 and the extinction value at 623 mμ the Mg^{++} content of an aliquot of the sample was calculated. This agreed with the Mg^{++} determined directly on the sample by the microcolorimetric method.

Identification of vinyl pheoporphyrin a_5 from *Chlorella* was made by comparing the spectrum with the synthetic crystalline compound derived from spinach. The agreement in extinction maxima and wave-lengths of the absorption bands was satisfactory. The vinyl pheoporphyrin a_5 compound from *Chlorella* was converted to vinyl chloroporphyrin e_6 trimethyl ester and its absorption spectrum agreed in band maxima and wave-lengths with that of the crystalline synthetic compound. The vinyl pheoporphyrin compound from *Chlorella* was reduced with palladium and hydrogen in acetic acid, and the shift in absorption bands supported the view that only one vinyl group was present; the spectrum agreed with that of synthetic vinyl pheoporphyrin reduced at the vinyl group in the same way and with the published data on pheoporphyrin a_5. The compound of *Chlorella* reduced at the vinyl group was converted to an oxime and spectrophotometrically matched the spectrum of the published curve of pheoporphyrin a_5 oxime.

Several pigments related to vinyl pheoporphyrin were observed in low concentrations, especially if the *Chlorella* was grown at a high temperature. These pigments appear to be decomposition products of vinyl

pheoporphyrin, possibly vinyl phylloerythrine and vinyl hydroxypheoporphyrin.

Confirmatory evidence supporting the structure of protochlorophyll as a phytyl ester was obtained in solubility studies of the compound isolated from etiolated barley seedlings.

Illumination of *Chlorella* 31 at low temperatures suggests that Mg vinyl pheoporphyrin is destroyed on exposure to light unless it is esterified with a phytol.

A microcolorimetric modification of the Titan yellow method is described for the determination of Mg^{++} in the range of 1 to 4 γ.

We desire to acknowledge the invaluable technical assistance of Miss Lorraine Blake in this study.

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CHEMICAL DETERMINATION OF HISTAMINE IN HUMAN BLOOD*

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The normal histamine content of human blood as determined by bioassay ranges from 1 to 8 γ per 100 ml., with an average value of about 4 γ (1, 2). These low levels in human blood have limited the development of a procedure for chemical analysis, although a chemical method applicable to blood would facilitate the accumulation of data from large groups of subjects.

The two chemical methods available for the analysis of histamine in biological fluids are not directly applicable to human blood. The simpler technique of Rosenthal and Tabor (3), which utilizes the non-specific coupling reaction of imidazoles with 4-nitroaniline, requires a specimen of at least 20 ml. In McIntire's method, the histamine is isolated from plasma under specified conditions (4) and the 2,4-dinitrofluorobenzene derivative is used for the determination (5). This procedure has the required degree of sensitivity but involves two counter-current extraction steps and a scaling down to micro levels for analysis of human blood.

By modifying the purification procedure of McIntire to make it applicable to whole blood filtrates without appreciable loss of histamine, and by scaling down the chemical method of Rosenthal to increase its sensitivity 5-fold, a procedure was developed for the colorimetric analysis of 0.1 to 1.0 γ of histamine in a 5 to 10 ml. specimen of whole blood.

EXPERIMENTAL

It has been reported that the histamine of the blood is concentrated in the leucocytes (6). Analysis of white cells or whole blood, therefore, seems preferable to plasma, and, of these, analysis of whole blood is the simpler procedure.

Prior to application of McIntire's method (4) for the isolation of histamine from plasma, it is necessary to precipitate the proteins in whole blood. Several protein precipitants were tested. Trichloroacetic acid gives the clearest filtrates but interferes with subsequent adsorption on the ion exchange medium. Histamine is precipitated by alkaline zinc, barium hy-

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droxide, and ethyl alcohol. Metaphosphoric acid filtrates contain a small amount of protein but they are satisfactory since the filtered protein is later precipitated during butanol extraction.

In McIntire's method for the purification and concentration of histamine from biological fluids advantage is taken of the fact that histamine is extracted from an aqueous solution by an equal volume of *n*-butanol containing at least 20 per cent by weight of salt at pH 12 or more. Under these conditions, and particularly in plasma, extraction is highly selective for histamine. When whole blood filtrates are treated similarly, other substances, particularly ergothioneine, are also extracted to some extent by butanol and these substances must be treated as interferences in the subsequent analysis.

Histamine is adsorbed from the butanol extract by an ion exchange medium which contains free carboxyl groups. McIntire proposed the use of cotton acid succinate. In our hands the cotton ester was found to vary in efficiency and, for quantitative recoveries, it was necessary to reduce the flow of the butanol solution through the column to an impractical rate. The substitution of Amberlite IRC-50, a resin of the carboxylic acid type which has similar adsorption properties, for the cotton ester was found to facilitate the isolation of histamine. The resin was found to be inefficient in column operation but quantitative adsorption was obtained in batch operation by shaking the resin beads with the butanol solution for a specified time. Neither cotton acid succinate nor Amberlite adsorbs histamine when more than trace amounts of inorganic salts are present. Contamination of the butanol extract by the aqueous phase must, therefore, be avoided. Prevention of losses during the elution step due to the solubility of histamine in water and the presence of traces of inorganic salts on the resin beads is achieved by washing the resin free of butanol in 95 per cent ethyl alcohol alone.

It was found that the quantitative elution of histamine from the Amberlite required the eluting acid to be in contact with the resin for 15 minutes. Subsequent rinsing of the acid with sodium nitrite, necessary for the chemical analysis, provided conditions for the complete elution of histamine. The separate elution step is necessary, since the resin itself otherwise releases a large amount of material which produces color during the subsequent chemical analysis.

Rosenthal's method (3) is sufficiently sensitive for detection of 0.5 γ of histamine in pure solution as the red azo compound which results from the coupling of 4-imidazole ethanol with the diazotate of 4-nitroaniline. For our purposes it was necessary to reduce the volumes and change the proportions of the reagents in order to obtain reproducible readings in the range from 0.1 to 0.5 γ.

Quantitative determination of these small amounts of histamine in whole blood is complicated by several sources of interference. The copper present in whole blood, presumably because it is bound to protein, does not interfere with the determination, although copper ion inhibits the coupling reaction. The fact that histamine added to intact whole blood is not recovered has been attributed to the buffer action of intact blood proteins on a wide variety of active substances, of which histamine may be one. It was found that the prior treatment of whole blood with a small amount of concentrated sulfuric acid permitted quantitative recoveries of added histamine. This acid was, therefore, added routinely to all blood specimens to prevent protein binding and to inhibit bacterial growth.

Other interfering substances which are extracted from whole blood filtrates by butanol are not removed by an initial extraction with ether and are also adsorbed by the ion exchange medium and eluted under the same conditions as histamine. These include large amounts of a number of blood extractives. Analysis of whole blood from the pig failed to reveal histamine, although at least one other compound, presumably ergothioneine, was extracted and gave appreciable color on analysis. Such interference is minimized by prolonged heating of the eluate with a higher concentration of nitrous acid than was originally suggested by Rosenthal.

Although butanol extraction removes some substances which interfere with the coupling reaction, and heating with nitrous acid minimizes the effect of other interfering substances present in the butanol extract, extraneous color still develops during the coupling reaction. Rosenthal found on analysis that of the substances in the blood filtrates which couple with 4-nitroaniline only the azo compound of histamine retains its red color at pH 7.7, while the other azo compounds range in color from yellow to orange at this pH. Blood which had been stored in the blood bank for 1 month and found to be free of histamine yielded a yellow azo compound at pH 7.7 on analysis. This yellow color showed no change over a pH range from 7.7 to 3.0. In contrast, solutions containing only the azo compound of pure histamine showed a color range from red to orange to yellow over a pH range of 7.7 to 4.0.

These color changes were utilized to correct for the extraneous color developed during the blood analysis. The tubes were read at pH 7.7, *i.e.* after equilibration with veronal buffer, at which only histamine produced a red color, and at pH 3.0, obtained by the addition of a measured drop of glacial acetic acid, at which all the reaction products were yellow. The difference between the optical densities of the two readings was assumed to be proportional to the amount of histamine present. Differences in optical density plotted against concentration of histamine standards gave good agreement with Beer's law. Both readings were made at a wave-length of 525 mμ, at which a maximum difference between red and yellow absorption occurs.

Reagents—All reagents must be made up in copper-free water.

1. Metaphosphoric acid, approximately 6 per cent. Prepare fresh each week and store in the refrigerator.[1]

2. Sodium nitrite, 4 and 1.6 per cent. Prepare fresh every week and store in the refrigerator.

3. 4-Nitroaniline, 2 per cent. Dissolve 2.0 gm. of 4-nitroaniline in 100 ml. of 4 per cent (by volume) HCl. Store in the refrigerator.

4. Diazonium working solution. Add 0.5 ml. of 4 per cent sodium nitrite to 5 ml. of cold nitroaniline solution in an ice bath immediately before use.

5. Sodium carbonate. 21.4 gm. of anhydrous salt per 100 ml. of solution.

6. Veronal buffer. 9.2 gm. of diethylbarbituric acid and 2.0 gm. of NaOH per liter, adjusted to pH 7.7 with molar acetic acid.

7. Dry salt mixture. Mix anhydrous sodium sulfate and tertiary sodium phosphate monohydrate (4) in a ratio of 6.25 to 1 gm.

8. Standard solutions. Solutions of histamine dihydrochloride in 3.8 per cent (by volume) HCl equivalent to concentrations of 0.2, 0.5, and 0.8 γ of free base per ml. These should be prepared fresh for each determination from a stock solution containing 1 mg. of free base per ml. in 3.8 per cent HCl. The stock solution may be stored for 1 month in the refrigerator.

9. HCl. 3.8 per cent by volume of concentrated (37.5 per cent) HCl.

10. Amberlite IRC-50.[2]

11. NaOH, 20 per cent.

12. *n*-Butanol, reagent grade. Redistil if a blank gives color with the diazonium reagent.

13. Methyl isobutyl ketone, commercial purified grade.

Procedure

It is necessary to use calibrated constriction pipettes for all measurements of volumes of 1 ml. or less. These may be made or may be purchased from the Microchemical Specialties Company, Berkeley, California.

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[1] The commercial c.p. acid in pellet or stick form contains a minimum of 25 per cent sodium phosphate and is not as effective a protein precipitant as acid prepared by dehydrating c.p. orthophosphoric acid. For the preparation of metaphosphoric acid 300 ml. of 85 per cent orthophosphoric acid are heated in a 1 liter graphite crucible until the water is drawn off and fumes of phosphorus pentoxide appear. The crucible is heated for 2 additional hours. The contents are poured into a metal container with a tight fitting cover and allowed to cool. This preparation does not solidify.

[2] Obtained through the courtesy of the Resinous Products Division, Rohm and Haas Company, Philadelphia.

Concentration of Histamine from Whole Blood—A 5 to 10 ml. sample of oxalated blood, collected under sterile precautions, is measured and delivered into a sterile tube. 1 drop (0.01 ml.) of concentrated sulfuric acid is added for each 2 ml. of blood and the contents are mixed. In this condition the blood may be analyzed immediately or stored at −15°. The blood is transferred to a 50 ml. round bottomed centrifuge tube and the storage tube is rinsed with an equal volume of water and 2 volumes of metaphosphoric acid. The rinsings are added to the blood, giving approximately a 1:4 dilution. After thorough mixing the tube is centrifuged at 2500 r.p.m. (radius 30 cm.) for 15 minutes, and the clear supernatant solution is poured into a 100 ml. round bottomed centrifuge tube equipped with a ground glass stopper. The solution is made alkaline to nitrazine paper with 2 to 5 ml. of 20 per cent NaOH, and 1 gm. of dry salt mixture is added for each 3.75 ml. of the supernatant solution. A volume of *n*-butanol equal to the total alkaline solution is added. The tube is stoppered and shaken mechanically for 30 minutes. After centrifugation at 1500 r.p.m. to separate the layers, most of the butanol layer is transferred to another glass-stoppered tube containing 400 mg. of dry Amberlite IRC-50. The aqueous layer is extracted a second time with butanol and the extracts are combined. Removal of the last traces of the second butanol layer is facilitated by the addition of several 5 ml. portions of fresh butanol. Contamination of the butanol extract by the aqueous phase should be avoided. The combined butanol extracts are shaken mechanically for 1 hour with the Amberlite. All of the Amberlite is then transferred to a 140 × 21 mm. tube with a narrowed section 25 × 9 mm. above a stop-cock and burette tip. The stop-cock opening is covered by a small plug of glass wool. The transfer is best accomplished by pouring the butanol through the tube, the separated butanol being used to rinse the Amberlite into the tube. The tube and Amberlite are rinsed once with about 40 ml. of 95 per cent ethyl alcohol which is drained by applying gentle suction to the tip of the tube. The stop-cock is closed and 1 ml. of 3.8 per cent HCl is delivered to the Amberlite and allowed to stand in contact with it for 15 minutes. The acid is drained dropwise into a 12 × 75 mm. Pyrex tube. The Amberlite is then rinsed with 1.0 ml. of 1.6 per cent sodium nitrite, which is also delivered into the Pyrex tube. The last drops of rinse solution are blown into the Pyrex tube.

Analysis of Histamine—The tube containing the Amberlite eluate is heated in an oven at 100° for 30 minutes. At this stage the tubes may be stoppered and stored overnight in the refrigerator, or chilled in an ice bath and analyzed immediately. To the cold Amberlite eluate in an ice bath, 0.2 ml. of the diazonium reagent is added with mixing, followed by 0.4 ml. of sodium carbonate. The contents are mixed and allowed to stand for

3 minutes. 0.25 ml. of methyl isobutyl ketone is pipetted into the tube and the contents are shaken vigorously thirty times, allowed to separate in the ice bath, and the tube is finally centrifuged. By means of a capillary pipette, the largest possible portion of uncontaminated ketone layer is transferred to another 12 × 75 mm. tube containing 1 ml. of veronal buffer measured by burette. The ketone is shaken with the buffer twenty times and allowed to stand in contact with it for 30 minutes in the refrigerator with occasional shaking. The ketone layer is then transferred to a 6 × 75 mm. colorimeter tube[3] and centrifuged if not perfectly clear. The tube is read in the Coleman junior spectrophotometer at a wave-length of 525 mμ against a blank prepared similarly with 1.0 ml. of 3.8 per cent HCl. For the standard curve, 1 ml. volumes of standard histamine solutions containing 0.2, 0.5, and 0.8 γ per ml. are carried through the analysis in duplicate at the same time. Following these readings, 1 measured drop

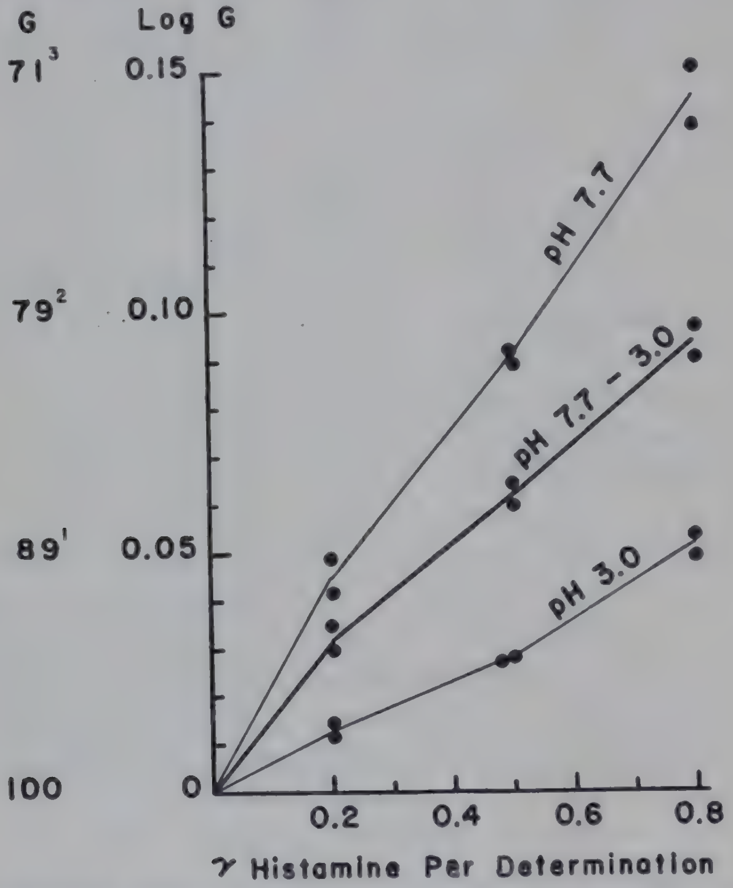

FIG. 1. Analysis of standard histamine solutions. *G* = galvanometer reading.

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[3] These tubes are made from Kimble hydrometer glass tubing stock, 6 mm. outside diameter, wall thickness less than 1 mm. to fit the Coleman adapter No. 6-110. The slit in this adapter is partially filled with Plicene cement so that a volume of 0.2 ml. of ketone may be read.

(3 to 5 c.mm.) of glacial acetic acid is added to each tube and the contents are mixed. The tubes are again read at 525 mμ. The concentration of histamine is proportional to the difference between the optical densities of the two readings.

TABLE I

Recovery of Added Histamine from 10 Ml. of Histamine-Free Blood

Added histamine	Found on analysis	Per cent recovery
γ	γ	
0.2	0.20	100
0.2	0.19	95
0.5	0.46	92
0.5	0.51	102
0.8	0.74	93
0.8	0.81	101
1.0	0.95	95
1.0	1.01	101

TABLE II

Chemical Determination of Histamine Levels in Human Blood

Subject	Age	Volume of specimen	Total histamine content	Histamine level	Comment
		ml.	γ	*γ per 100 ml.*	
HN	Adult	10	0.16	1.6	Normal
		10	0.14	1.4	"
NE	"	9	0.40	4.4	"
		9	0.42	4.6	"
RL	"	7	0.30	4.3	"
		7	0.26	3.7	"
PG	"	10	0.06	0.6	"
		10	0.08	0.8	"
760	"	10	0.27	2.7	Asthma
	yrs.				
750	1½	7	0.49	7.0	Infantile eczema
751	2½	9	0.82	9.1	" "
752	11	9.5	0.07	0.7	Normal
823	9½	12	0.42	3.5	"

Results

In Fig. 1, the curve obtained on analysis of standard solutions of histamine is presented together with the logarithms of the colorimetric readings at pH 7.7 and 3.0.

In Table I the percentage recovery of standard solutions of histamine from 10 ml. of acidified blood is given. The blood used had been stored for at least 1 month and had been found to be free of histamine on analysis. Recoveries ranged from 92 to 102 per cent.

In Table II, blood histamine levels in micrograms per 100 ml. of blood are given for nine subjects. The four duplicate determinations show a reproducibility within 10 per cent. Of the nine determinations, the two values higher than 5 γ per 100 ml. were obtained in children with infantile eczema whose white cell counts were at the upper limit of normal. The significance of the wide range in levels below 5 γ per 100 ml. is unknown.

DISCUSSION

In a limited series of subjects, a wide range in blood histamine was found (0.1 to 9.1 γ per 100 ml.). The values obtained for normal subjects fell within the lower limits of normal reported by bioassay (1); two patients with active allergy had blood levels at the upper limit of the normal. These findings are consistent both with the reported results of bioassay and with the observation that patients with allergic manifestations have high blood histamine levels (7). A study of the blood histamine levels in a large series of children with various conditions is now in progress.

Probably whole blood histamine alone is measured by the proposed method. It is less sensitive but more easily reproducible than methods based on bioassay. With careful technique, its reproducibility is within 10 per cent. The lack of analytical precision is compensated for in part by the fact that the chemical analysis permits storage of specimens over a long period of time and the procedure is less time-consuming.

SUMMARY

The histamine purification method of McIntire and the chemical method of Rosenthal and Tabor have been modified to permit the analysis of 0.1 to 1.0 γ of histamine in human blood samples of 5 to 10 ml., with an error of 10 per cent. The levels obtained by this method in a small group of subjects are reported.

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THE EFFECT OF A SERIES OF FLAVONOIDS ON HYALURONIDASE AND SOME OTHER RELATED ENZYMES*

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Among the theories which have been advanced for the possible mechanism of action of vitamin P substances in regulating capillary resistance, it has been proposed (1) that they may act as inhibitors of hyaluronidase. As evidence that this enzyme may play a part in capillary permeability, the capillary wall and supporting tissue have been reported (2, 3) to contain metachromatic staining material, either hyaluronic acid or chondroitin, both of which are subject to attack by hyaluronidase. Duran-Reynals (4) showed that the enzyme increased the permeability of the vascular system, and Chambers and Zweifach (5) found petechial hemorrhages following injection of hyaluronidase in mesentery preparations.

The effect of a limited number of flavonoids on the action of hyaluronidase *in vitro* was studied by Beiler and Martin (1) and by Clark and Geissman (6). Levitan (7) and Elster (8) have reported on the action of rutin on hyaluronidase *in vivo*. The present report deals with the effect of thirteen flavonoids on hyaluronidase, with two methods *in vitro* and one test *in vivo*, in a study of the relation of structure to inhibitory activity. In addition, the action of these compounds on some other enzymes involved in theories of vitamin P activity is reported in order to determine the specificity of the inhibitory activity.

Streptococcal hyaluronidase differs from the bovine enzyme in its sensitivity to pH changes, optimum pH of 7.0, and greater release of reducing substances from the substrate (9). It was chosen for study as being closely related to the bovine enzyme. β-Glucuronidase, which releases glucuronic acid from conjugates but has no action on hyaluronic acid, was also studied with the flavonoids. Lysozyme, an enzyme which liberates ketohexose and hexosamine from polysaccharides of certain bacteria but not from hyaluronic acid, was used as a third related enzyme. In addition, the effects of the compounds on the oxidation of epinephrine by mushroom tyrosinase were investigated, since inhibition of epinephrine oxidation has been suggested as the basis for vitamin P action (10).

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Materials and Methods

Inhibitors—The thirteen flavonoids which were studied[1] differed in the state of oxidation, the number of hydroxyl groups, the sugar components, and the methoxyl groups. Suspensions of 1 mg. of flavonoid per ml. of M/15 phosphate buffer, pH 7.5, were shaken, left at room temperature for 24 hours, centrifuged if necessary, and the concentration determined by the spectrophotometric method. The solutions were diluted with buffer to a concentration of 1×10^{-4} mole per ml., except in the case of rhamnetin, which was soluble only to the extent of 3×10^{-5} mole per ml. Dilutions of quercetin and xanthorhamnin were made to compare with this, since their structural relationship was important.

These solutions were tested by both *in vitro* methods. In addition, in the viscosity test, in which a much smaller volume of inhibitor and enzyme could be used, they were dissolved in 1 per cent tris(hydroxymethyl)aminomethane (TAM). Control experiments showed that this solvent with pH 9.0 had little effect on the test. For the work *in vivo* the flavonoids were injected at pH 8.0 or in 1 per cent TAM with appropriate controls.

Bovine Hyaluronidase—The enzyme was prepared by the Madinaveitia method (11) and contained 200 viscosity reducing units per mg. (400 turbidity reducing units per mg.). The substrate, hyaluronic acid, was prepared from umbilical cords by the Dorfman method (12). It contained 13.5 per cent glucosamine, 45.5 per cent reducing substances in terms of glucose, and 3.0 per cent nitrogen.

For testing the effect of the flavonoids on viscosity reduction by the enzyme, the Haas method (13) was used; 100 viscosity reducing units of enzyme were incubated 1 hour with 10 ml. of flavonoid solution, and enzyme diluted with the same amount of solvent was used as a control. After determining the initial flow time of 4 ml. of substrate (1.5 mg. per ml. of Haas buffer) in an Ostwald viscosimeter at 25°, 0.1 ml. of the enzyme solution was added to the substrate, and at 2 minute intervals the flow time was redetermined. The inhibition was calculated from the formula,[2] per cent inhibition $= 100 \times 1 - y - x/y - z$, where $y =$ the initial flow time of enzyme, substrate, and inhibitor, $x =$ the flow time after 10 minutes for the enzyme, the substrate and inhibitor, and $z =$ the flow time after 10 minutes for the enzyme and the substrate. Additional controls were run with appropriate dilutions of the flavonoid, substrate, and buffer.

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[1] Dihydroquercetin, eriodictyol, homoeriodictyol, rhamnetin, and xanthorhamnin were supplied by Dr. W. G. Bywater of S. B. Penick and Company. Naringin, hesperidin, and hesperidin methyl chalcone were supplied by the California Fruit Growers Exchange. The remaining flavonoids studied were prepared by one of us (L. M. W.).

[2] It was not possible to determine inhibition by the formula $(100 \times R - R_0)/R$, since at the concentrations of inhibitors used, the point of the half viscosity could not be reached with many of the compounds.

A modification of the Dorfman method (12) was used for testing the effect of the inhibitors on the turbidity-reducing action of the enzyme. 10 units of the hyaluronidase in 0.5 ml. of 0.9 per cent saline were mixed with 0.5 ml. of the flavonoid solution and let stand 1 hour. 1 ml. of substrate containing 0.2 mg. of hyaluronic acid in 0.1 M acetate buffer at pH 6.0 was added, and the mixture was incubated 30 minutes at 37°. After heating the tubes at 60° for 10 minutes, 8 ml. of 0.1 per cent Fraction V bovine albumin in acetate buffer at pH 3.75 were added to develop the turbidity of the residual hyaluronic acid, and the tubes were read in a Fisher electrophotometer at 525 mμ. Controls were run with inhibitors and substrate, and the per cent inhibition was calculated by comparison with the activity of the enzyme and the substrate alone.

The *in vivo* method was that of Humphrey (14), with modifications according to Hechter and Sully (15). Ten 300 gm. white guinea pigs were injected 4 hours before the test with flavonoids dissolved at pH 8.0 in an amount, or a fraction thereof, equimolar with 100 mg. of rutin. The animals were shaved and injected on each side with 0.2 ml. of a control solution of hemoglobin and saline, prepared by hemolyzing packed rabbit erythrocytes with 5 volumes of water, centrifuging, and diluting 1:1 with 0.9 per cent saline. They were also injected on each side with 0.2 ml. of hemoglobin saline containing 0.01 unit of hyaluronidase. Normal animals were similarly treated. After 20 minutes they were killed, and the areas of spread on the inner surface of the skin were calculated by multiplying the product of two diameters measured at right angles by $\pi \div 4$. The average values for the spread with saline were subtracted from those with enzyme, and the per cent inhibition was determined by comparison with the values for normal animals.

Streptococcal Hyaluronidase—The enzyme was prepared from filtrates of streptococcus type B grown in Difco brain-heart infusion for 4 days at 37°, and fractionated at pH 7.2 with $(NH_4)_2SO_4$. The fraction between 35 and 70 per cent of saturation was dialyzed and lyophilized to yield material with 15 viscosity-reducing units per mg. The hyaluronic acid used as the substrate was prepared by the method given previously; the testing procedures and solutions of the flavonoids were the same as in the studies with the bovine enzyme.

β-Glucuronidase—The procedure of Graham (16) was followed for the preparation of the enzyme, which contained 1700 units per mg. of N, as determined by the method of Fishman, Springer, and Brunetti (17) with phenolphthalein glucuronide as the substrate.

Solutions of the flavonoids in M/15 phosphate buffer were diluted to

1×10^{-4} mole per ml. with water, and to 1 ml. of these solutions 0.1 ml. of 1:10 glucuronidase and 0.8 ml. of acetate buffer, pH 4.5, were added. They were incubated at room temperature for 30 minutes and 0.1 ml. of 0.01 M phenolphthalein glucuronide solution was added. After incubating 1 hour at 37°, the reaction was stopped by addition of 2.5 ml. of 0.4 M glycine buffer at pH 10.5. To the control tubes 1 ml. of the inhibitor solutions was then added, and after 1 minute they were read in the Fisher colorimeter at 525 mμ and compared with the standard phenolphthalein curve in the usual way.

Lysozyme—Crystalline lysozyme was used for the study; the substrate was a suspension of *Micrococcus lysodeikticus* cells (18). To 4 ml. of this suspension 1 ml. of the flavonoid solutions (1×10^{-4} mole per ml.) was added and the initial turbidity was determined in the Fisher electrophotometer at 525 mμ. A 0.2 ml. sample of lysozyme, 0.2 mg. per ml. of 0.85 per cent saline, was then introduced, and the contents of the tube were mixed by inverting ten times. The decrease in optical density in 2 minutes was then estimated and compared with blank determinations containing no flavonoids.

Epinephrine Oxidation by Tyrosinase—The enzyme was prepared from mushrooms by the method of Parkinson and Nelson (19) and contained 4 *p*-cresolase units per mg. It was dissolved in water, 2 mg. per ml., and to 1 ml. of enzyme in Warburg vessels 1 ml. of the flavonoids (1×10^{-4} mole per ml.) was added. After equilibration 0.5 ml. of 0.02 M epinephrine was added. Controls were run with flavonoid and enzyme and flavonoid and epinephrine, as well as with epinephrine and enzyme and epinephrine alone. Flasks were run in duplicate and the experiments were carried out in air at 37°. The per cent inhibition was determined after subtracting the small values due to autoxidation of the epinephrine and epinephrine-flavonoid mixtures and to the oxidation of the flavonoid by the enzyme, so that the values obtained represented the true inhibition of the enzyme.

Results

The effects of the flavonoids on hyaluronidase are shown for the four tests in Table I. The results of the *in vitro* tests indicate that the aglucones are more inhibitory than the glucosides, since quercetin was more active than rutin or quercetrin, rhamnetin more active than xanthorhamnin, and naringenin more active than naringin. A methyl group blocking hydroxyls in the 3′ position reduced the activity; homoeriodictyol was much less inhibitory than eriodictyol. However, a methoxyl group in the 7 position had little effect, since rhamnetin and quercetin were equally active. Hydroxyls in both the 3′ and 4′ positions produced greater activity than a single hydroxyl, as evidenced by the greater activity of eriodictyol compared with naringenin. The presence of a ketone group did not appear to be essential.

With increased alkalinity (as in TAM), activity was shown by some compounds which were inactive at lower pH. It is known that an increased oxidation of catechol takes place in alkali, and it was observed that aging the solutions in air produced increased activity and a darkening of color in many instances, so that the state of oxidation has considerable influence.

TABLE I

Effect of Flavonoids on Bovine Hyaluronidase

Compound	Per cent inhibition *in vitro*			Inhibition *in vivo*
	Turbidity, pH 7.5	Viscosity		Compared with rutin, mole basis
		pH 7.5	1 per cent TAM	
Rutin	0	0	54	1.0
Quercetrin	0	5	66	0.8
Quercetin	33, 17*	44, 20*	65	1.0
Dihydroquercetin	44	37	64	2.6
D-Catechin	88	70	73	6.3
Eriodictyol	100	85	85	2.0
Homoeriodictyol	0	0	23	0.0
Xanthorhamnin	0, 0*	0, 0*	23, 10*	0.5
Rhamnetin	11*	12*	22*	0.3
Naringin	0	0	14	0.8
Naringenin	6	0	35	
Hesperidin	0	0	0	0.0
" methyl chalcone	0	0	0	

* 3×10^{-5} mole per ml.

In the *in vivo* test the aglucones had no greater activity than the glucosides, as might be expected, since the sugar components could easily be removed. Here also the compounds most active *in vitro* and most susceptible to oxidation showed the greatest inhibition. Several compounds which were inactive *in vitro* showed activity *in vivo*, which would be compatible with the theory that activation by oxidation in the tissues could take place.

In an attempt to clarify these *in vivo* results, the effect of some tissues and enzymes on the activation of the inhibitory action of these compounds was studied. A suspension of 100 mg. of rutin was incubated 24 hours with hog pancreas homogenate (1 gm. per 10 ml. of M/15 phosphate buffer, pH 7.5). After removal of protein by heating 20 minutes, followed by centrifuging, the supernatant was tested for inhibitory activity. Appropriate controls with heated pancreas homogenate and heated rutin were also run. Some slight evidence of activation was found, but the results were very variable, and similar experiments with pancreatin, taka-diastase, emulsin, and muscle homogenate gave negative results. However, when liver, spleen, and blood were tested under the same conditions as those described for pancreas homogenate, slight but constant activation was produced (Table II).

Since it is known that catechol and related compounds are rapidly oxidized by peroxidase in the presence of traces of H_2O_2 in tissues, the effect of a purified preparation of this enzyme was studied. The enzyme was isolated from horse-radish and in solution contained 0.2 mg. of N per ml.; 1 ml. was equivalent to 1.75 mg. of purpurogallin. 2 ml. of fresh solutions of the inhibitors were treated with 0.01 ml. of 1:100 H_2O_2 and 0.1 ml. of peroxidase for 30 minutes. The reaction was stopped by the addition of 0.5 ml. of 2 N H_2SO_4. After neutralizing and warming to remove any residual peroxide, they were tested for inhibitory action by both of the *in vitro* methods, with appropriate controls. The results are shown in Table III. Definite activation of naringenin, rutin, and dihydroquercetin was produced, but none for hesperidin.

TABLE II

Effect of Tissues or Enzymes on Rutin Inhibition of Hyaluronidase

Tissue or enzyme	Per cent inhibition
Pancreas	19, 0, 7, 0
Pancreatin	0, 0
Taka-diastase	0, 0
Emulsin	0, 0
Muscle homogenate	0, 0
Liver "	10, 17
Spleen "	15, 25
Whole blood	20, 25
Peroxidase	50

The results of the study of the action of flavonoids on the other systems are shown in Table IV. It is apparent that those compounds most inhibitory to bovine hyaluronidase were also most active against the four enzymes investigated here, while those with least activity against the bovine enzyme had negligible inhibitive effect in these systems. Some difference in degree of action can be observed: β-glucuronidase was inhibited to some extent by all the compounds tested, though the greatest action was shown by quercetin, D-catechin, dihydroquercetin, and eriodictyol, as with the other systems. The streptococcal hyaluronidase also was most strongly affected by these compounds.

TABLE III

Effect of Peroxidase on Flavonoid Inhibition of Hyaluronidase

Compound	Treatment	Per cent inhibition	
		Turbidity	Viscosity
Rutin		0	0
	Peroxidase	54	16
Dihydroquercetin		30	42
	Peroxidase	100	67
Naringenin		0	0
	Peroxidase	50	27
Hesperidin		0	0
	Peroxidase	0	0

TABLE IV

Effect of Flavonoids on Enzymes

Compound	Per cent inhibition				
	Streptococcal hyaluronidase		β-Glucuronidase	Lysozyme	Tyrosinase
	Turbidity	Viscosity			
Rutin	0	0	28	0	17
Quercetrin	0	0	15	0	
Quercetin	90	39	38.9*	15	11
Dihydroquercetin	100	36	51	36	72
D-Catechin	100	73	39	64	35
Eriodictyol	100	59	26	33	41
Homoeriodictyol	0	6	6	0	0
Xanthorhamnin	0.0*	0.0*	14.3*	6.4*	
Rhamnetin	0*	0*	15*	15*	
Naringin	0	0	7	0	7
Naringenin	7	0	28	10	31
Hesperidin	0	0	13	0	0
" methyl chalcone	0	0	25	0	

* 3×10^{-5} mole per ml.

DISCUSSION

Recently James and coworkers (20) have shown that catechol, with free orthohydroxyl groups, is oxidized to the orthoquinone form with the introduction of a third hydroxyl group, active in forming color complexes and reacting with amino groups of protein. Resorcinol, with metahydroxyl groups, does not undergo this reaction. In the case of naringenin the introduction of a hydroxyl group in the 3′ position, already present in rutin and dihydroquercetin, would lead to quinone formation and further oxidation, but with hesperidin the probable reactive position 5′ would place hydroxyls in the meta position. This presents a possible explanation of the oxidative activation of the inhibitory action on hyaluronidase for those flavonoids with structures capable of forming orthoquinones. A similar reaction has been discussed by Bartlett (21) in connection with the inhibition of succinoxidase by the action of some orthoquinones on sulfhydryl groups of the enzyme. Hyaluronidase is inhibited by *p*-chloromercuribenzoate, a sulfhydryl-reactive compound, and contains free amino groups, so that it should be susceptible to the reactions discussed.

The *in vivo* results may be explained on the basis of oxidation by tissues and blood. In addition to other oxidative systems blood contains peroxidase; spleen and liver are blood-rich organs and these tissues were found to have an activating effect on rutin. The erratic results with pancreas may be explained by variations in the amount of blood present in the preparation.

From the results obtained in the studies of the action of the flavonoids on five enzymes, it appears that all of the enzymes were inhibited by those compounds capable of orthoquinone formation and that inhibition by the flavonoids is not specific, but probably due to reaction with amino or thiol groups in the protein molecule.

SUMMARY

Thirteen flavonoids were tested for their inhibitory action on bovine hyaluronidase by *in vitro* and *in vivo* methods. For maximum activity *in vitro* the aglucones were more effective than the glucosides, and free hydroxyl groups in both the 3′ and 4′ positions were necessary. Activation by some tissues, by increased pH, and by oxidation such as by peroxidase, may explain the *in vivo* activity of many of the flavonoids against hyaluronidase.

The inhibitory action of these flavonoids on streptococcal hyaluronidase, β-glucuronidase, lysozyme, and tyrosinase oxidation of epinephrine in general followed the structural pattern observed with bovine hyaluronidase and indicated that the inhibition is not specific.

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SYNTHESIS OF HEME IN VITRO BY THE IMMATURE NON-NUCLEATED MAMMALIAN ERYTHROCYTE*

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When the whole blood of subjects with sickle-cell anemia is incubated aerobically with N^{15}-labeled glycine, labeled heme is formed (2). This finding demonstrates the synthesis of heme from glycine *in vitro.* Significant synthesis of heme is not observed when normal human blood is incubated under similar conditions. This difference could be due to the presence of numerous immature cells in the blood in sickle-cell anemia. However, in the initial experiments previously reported, in which the capacity for *in vitro* synthesis of heme from blood samples from other subjects with hematologic disorders, characterized by elevated reticulocyte counts, was studied, significant heme synthesis was not observed. It was clear, therefore, that, although the reticulocytes in the blood of sickle-cell anemia subjects might be involved in the heme synthesis observed, the mere presence of numerous reticulocytes does not insure the ability of blood to synthesize heme *in vitro*. These studies have been extended further to a number of disorders in subjects with elevated reticulocyte counts in the peripheral blood. The results of these studies, to be reported in detail later, confirm the finding that some blood samples with elevated reticulocyte counts do not perform significant heme synthesis *in vitro;* they reveal, however, that the *in vitro* synthesis of heme in human blood is not restricted to the blood of sickle-cell anemia.

In order to investigate some of the problems posed by these observations, further information on the rôle of the reticulocyte in the synthesis of heme was sought. It was desirable to determine the capacity for synthesis of heme of reticulocytes in the blood of experimental animals which had no previous hematologic disorder. This report is concerned with the capacity for heme synthesis of blood samples drawn from rabbits in which reticulocytosis was produced in response to bleeding or phenylhydrazine hemolysis.

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EXPERIMENTAL

Normal rabbits were bled by cardiac puncture. 20 to 30 ml. of blood were removed three to five times a week and equivalent volumes of normal saline were injected intraperitoneally. Reticulocyte counts were performed on smears of blood drawn from ear veins and stained with brilliant cresyl blue; 1000 erythrocytes were counted for each determination of the percentage of reticulocytes.

In a typical experiment, 20 to 30 ml. of blood were added to a flask containing heparin, 50 mg. of glycine labeled with 32 atom per cent excess N^{15}, and 1 to 2 mg. each of crystalline penicillin and streptomycin to inhibit bacterial contamination. The flasks were incubated for 24 hours at 37°; the blood was aerated with a 95 per cent O_2-5 per cent CO_2 mixture. At the end of the period of incubation, the blood was centrifuged and the serum removed. Hemin was prepared from the red cells by the usual procedure (3), recrystallized (4), and its N^{15} concentration determined.

Similar experiments were performed with the blood of rabbits in which reticulocytosis was produced by acetylphenylhydrazine. In most instances 60 mg. of acetylphenylhydrazine were injected subcutaneously in one dose several days prior to withdrawal of the blood sample tested. In Experiments 80-2, 80-3, 80-5, 80-9 in Table II, 25 mg. of acetylphenylhydrazine were injected daily for 6 days and blood samples were drawn on the 7th day.

The effect of anaerobiosis was studied in Experiment 74. For 45 minutes prior to the addition of N^{15}-labeled glycine and for 15 minutes thereafter a mixture of 95 per cent N_2-5 per cent CO_2 was passed through the flask containing the blood sample. The flask was then sealed. The procedure was otherwise identical with that described for the aerobic experiments.

Synthesis of heme by erythrocytes washed free of plasma was investigated in Experiment 80-3 (Table II). After an initial separation of the erythrocytes from the plasma by centrifugation, the cells were washed twice with physiologic saline and were then suspended in 15 ml. of physiologic saline. The incubation with labeled glycine was then carried out as described above.

The capacity for synthesis of heme of erythrocytes hemolyzed by saponin was tested in Experiments 72 and 80-5. In Experiment 72, the erythrocytes were separated from the plasma by centrifugation, washed once with physiologic saline, and then mixed with a solution of 200 mg. of saponin dissolved in 15 ml. of physiologic saline. Hemolysis was complete within 15 minutes. In Experiment 80-5, the erythrocytes were washed twice with physiologic saline and were then mixed with a solution of 37.5 mg. of saponin dissolved in 15 ml. of physiologic saline. Hemolysis was complete within 15 minutes. Aerobic incubation with N^{15}-labeled glycine was then performed in the same manner as that described for the intact cell system.

To determine whether high oxygen tension was essential for significant synthesis of heme in this *in vitro* system, in Experiment 80-9 the incubation was performed with the flask open to air.

RESULTS AND DISCUSSION

Blood from normal rabbits with reticulocyte counts ranging from 0.0 to 5.9 per cent yielded hemin with concentrations of 0.006 to 0.036 atom per cent excess N^{15} (Table I). Blood samples from rabbits in which reticulocytosis was induced by bleeding or phenylhydrazine administration, however, yielded hemin with much higher concentrations, 0.094 to 0.307 atom per cent excess N^{15} (Table II). Inasmuch as the glycine was labeled with 32 atom per cent excess N^{15}, and it is reasonable to assume that the N^{15} concentration in the newly formed heme is of similar magnitude, the isotope values in the hemin (Table II) reveal that approximately 0.3 to 0.9 per cent of the total heme in the *in vitro* system was newly synthesized during the 24 hour period of incubation.

TABLE I

Blood from Normal Rabbits

25 ml. of blood were incubated in an atmosphere of 95 per cent O_2-5 per cent CO_2 at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N^{15}.

Experiment No.	Reticulocytes	N^{15} concentration in hemin
	per cent	*atom per cent excess*
39	0.8	0.011
70-1	1.4	0.015
75-1	2.0	0.006
75-2	3.5	0.011
75-3	1.7	0.016
73-2	2.1	0.014
73-1	0.0	0.029
84-1	5.9	0.023
84-2	4.0	0.036
84-3	2.5	0.032
85-2	1.7	0.018
85-3	1.8	0.023
85-4	1.9	0.020

These findings demonstrate that the non-nucleated but immature mammalian erythrocyte is capable of synthesizing heme *in vitro*. This capacity for heme synthesis is present to a significant degree in rabbit blood characterized by an increased number of reticulated cells, whereas significant synthesis does not occur with normal mature erythrocytes.

The data in Table III indicate the effect of repeated bleeding on the reticulocyte counts and the capacity for heme synthesis. Elevated reticulocyte counts are associated with appreciable concentrations of N^{15} in hemin but no close correlation between the height of the reticulocyte count and the hemin isotope concentration exists. The findings in Experiments 75-1 and 75-3 (Table III) are of interest. Blood from normal rabbits with reticulocyte counts of 1.7 and 2.0 per cent yielded N^{15} values in hemin of insignificant concentration. In Experiment 75-1, 2 days after the withdrawal of 25 ml. of blood, a N^{15} concentration in hemin of 0.056 atom per cent excess was achieved with a reticulocyte count of only 1.5 per cent, and in Experiment 75-3, 4 days after the first bleeding and 2 days after a second bleeding, with a reticulocyte count of 1.7 per cent the N^{15} concentration in hemin was 0.088 atom per cent excess. These isotope concentrations represent significant synthesis of heme. Two possible explanations of these results in the presence of normal reticulocyte counts may be offered. (*a*) The capacity of reticulocytes for synthesis of heme may vary widely. It

TABLE II

Rabbit Blood with Reticulocytosis

25 ml. of blood were incubated in an atmosphere of 95 per cent O_2-5 per cent CO_2 at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N^{15}.

Experiment No.	Method of producing reticulocytosis	Reticulocytes	N^{15} concentration in hemin	Remarks
		per cent	*atom per cent excess*	
33	Bleeding	35.0	0.178	
45	"	17.4	0.094	
58	"	15.1	0.236	
59	"	8.2	0.182	
60	"	24.9	0.269	
62	"	8.6	0.140	
65	Acetylphenylhydrazine	38.6	0.196	
67-2	"	39.0	0.148	
80-2	"	88.3	0.221	
80-9	"	67.3	0.272	Incubated in air
74	"	37.8	0.016	" " 95% N_2-5% CO_2 atmosphere
80-3	"	88.7	0.307	Cells suspended in saline
72	"	23.0	0.002	Hemolyzed with saponin
80-5	"	61.6	0.004	" " "
93	"	82.6	0.007	" " distilled water

is reasonable to assume that this capacity is a function not only of the number of reticulocytes but of their age and morphologic development. (*b*) Synthesis of heme may be performed *in vitro* not only by reticulocytes but by other immature erythrocytes which, however, are not stained by brilliant cresyl blue.

Table III

Effect of Repeated Bleeding on Rabbits

25 ml. of blood were incubated at 37° for 24 hours with 50 mg. of glycine labeled with 32 atom per cent excess N^{15}. In Experiment 75-1 bleedings of 25 ml. each were made on 0, 2, 4, 7, and, 10 days; in Experiment 75-3 bleedings of 25 ml. were made on 0, 2, 4, and 7 days; in Experiment 85-2 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, 6, and 7 days; in Experiment 85-3 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, 6, and 7 days; in Experiment 85-4 bleedings of 25 ml. were made on 0, 1, 2, 4, 5, and 6 days.

Experiment No.	Day	Reticulocytes	N^{15} concentration in hemin
		per cent	*atom per cent excess*
75-1	0	2.0	0.006
	2	1.5	0.056
	7	5.0	0.023
	10	19.5	0.096
75-3	0	1.7	0.016
	4	1.7	0.088
	7	10.0	0.177
85-2	0	1.7	0.018
	1	2.7	0.027
	6	20.0	0.125
	7	21.1	0.114
85-3	0	1.8	0.023
	1	3.3	0.029
	2	5.5	0.128
	4	13.8	0.109
	6	24.7	0.084
	7	25.1	0.152
85-4	0	1.9	0.020
	1	2.6	0.031
	2	3.8	0.033
	4	9.0	0.109
	6	23.7	0.057

It has been generally agreed that, although the reticulocyte is an immature erythrocyte, the deposition of hemoglobin is completed prior to denucleation (5, 6). The demonstration that the non-nucleated immature erythrocyte is capable of synthesizing heme *in vitro* raises the possibility that a significant, although perhaps small, proportion of heme formation occurs *in vivo* in the non-nucleated immature cell in the bone marrow and in the peripheral blood.

To consider the *in vitro* synthesis of heme as evidence of *in vitro* hemoglobin formation, one must assume that the immature non-nucleated erythrocyte contains globin or is capable of synthesizing globin and that coupling of heme to globin can occur in the erythrocyte at this stage of its development. Evidence obtained in studies with duck erythrocytes suggests that globin can be synthesized *in vitro*.[1] The finding of synthesis of heme and of the probable synthesis of hemoglobin by the non-nucleated immature cell represents a departure from the general belief that hemoglobin formation is completed prior to the disappearance of the nucleus from the red cell.

It appears, on the basis of the available evidence and on the assumptions which have been noted, that a significant but minor part of hemoglobin synthesis is carried out by the non-nucleated immature red cell. The ability of this cell to synthesize heme and, probably, hemoglobin is pertinent to the problem of the time relationships between hemoglobin deposition in the red cell and release of the red cell into the circulation. Earlier studies (7) in normal humans and in patients with hematologic disorders have shown that red blood cells containing N^{15}-labeled heme appear in the peripheral blood within several hours after the oral administration of N^{15}-labeled glycine. These studies suggest that the human erythrocyte is released into the circulation at a time which approximates the completion of hemoglobin deposition in the cell. If the red cell entering the circulation is mature, hemoglobin deposition is probably completed shortly before release of the cell from the bone marrow. This view is supported by the absence of significant heme synthesis *in vitro* by normal human blood (2). If, however, the red cell entering the circulation is immature, hemoglobin deposition may not yet be complete and further hemoglobin synthesis may occur in the peripheral blood.

In vitro synthesis of heme by reticulocytes offers an alternative explanation for increases in free erythrocyte protoporphyrin noted by Watson *et al.* (8) on incubation of human and rabbit blood samples containing immature erythrocytes. These increases were considered by these authors to be the result of intracorpuscular degradation of hemoglobin. It is possible that the increase in protoporphyrin represents at least in part an actual synthesis of new protoporphyrin *in vitro*.

No significant synthesis of heme occurred under the anaerobic conditions described (Experiment 74 in Table II) nor with the cells hemolyzed by saponin (Experiments 72 and 80-5) or distilled water (Experiment 93). Satisfactory synthesis of heme was observed on incubation of whole blood in air (Experiment 80-9).

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[1] Shemin, D., London, I. M., and Rittenberg, D., *J. Biol. Chem.*, **183**, 757 (1950).

SUMMARY

The immature non-nucleated rabbit erythrocyte is capable of synthesizing heme *in vitro*.

We wish to acknowledge the valuable assistance of Miss Martha Yamasaki and to thank Mr. I. Sucher for the isotope analyses.

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THE SYNTHESIS OF PROTOPORPHYRIN IN VITRO BY RED BLOOD CELLS OF THE DUCK*

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Synthesis of heme has been shown to occur *in vitro* with the nucleated red blood cells of the duck (1). Incubation of duck blood with N^{15}-labeled glycine results in the formation of N^{15}-labeled heme. The *in vitro* system offers many biological and technical advantages for a study on the biosynthesis of porphyrins. The synthesis is rapid, the blood of birds is readily available, and large amounts can be obtained which permit comparative studies. The *in vitro* system, as compared to the whole animal, requires relatively small amounts of labeled compounds which are slightly diluted.

This communication will describe the use of the red blood cells of the duck as a system for the study of the biosynthesis of the porphyrin, heme.

EXPERIMENTAL

Incubation Experiments—Adult ducks were exsanguinated, without anesthesia, by cutting the exposed jugular veins and collecting the blood in a vessel containing 10 to 20 mg. of heparin. The heparinized blood was filtered through several layers of cheese-cloth to remove any débris, and about 3 mg. each of sodium benzyl penicillin and streptomycin calcium chloride complex were added to each 100 ml. of blood. Usually about 150 to 250 ml. of blood were obtained. Approximately 20 ml. of blood were used for each experiment. The effect of each factor or condition on heme synthesis was determined by comparison with the heme synthesis observed in a control blood sample. In most experiments the compounds were added without attempting to maintain isotonicity, while in some cases the substances were added as isotonic solutions. These conditions will be indicated in individual experiments. The glycine employed contained 32 atom per cent excess N^{15}.

The blood samples were incubated with shaking at 37°. Usually little or no hemolysis occurred as judged by the appearance of the plasma following incubation. A few samples in which hemolysis had occurred were discarded.

Hemin Isolation—After incubation the blood samples were centrifuged,

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plasma was removed, and a volume of water equal to that of the red cells was added. The red cell suspension was homogenized for 5 to 10 minutes in a Waring blendor. The crude hemin, prepared by the usual method (2, 3) from 20 ml. of blood, was stirred with 0.5 ml. of pyridine. After 5 to 10 minutes 4 ml. of chloroform were added and the solution was filtered. 15 ml. of acetic acid, saturated with sodium chloride, were added to the filtrate and the mixture heated to 105°. 0.1 ml. of concentrated hydrochloric acid was then added and the hemin was allowed to crystallize at room temperature for 24 hours. The crystals were separated by centrifugation and washed successively with 50 per cent acetic acid, water, alcohol, and ether (3, 4). The hemin was analyzed for its isotopic content in the usual manner.

Synthesis of Heme from Glycine in Vitro—To each of four 20 ml. samples of whole duck blood, 200 mg. of isotopic glycine were added. The blood was incubated at 37° for 24 hours in an atmosphere of 95 per cent O_2-5 per cent CO_2. The hemin isolated was found to contain 0.126 atom per cent excess N^{15}. This hemin was converted by the method of Grinstein (5) to protoporphyrin IX dimethyl ester and purified by chromatography and recrystallization. The N^{15} concentration of this compound, 0.124 atom per cent excess N^{15}, was, within the experimental error, the same as for the hemin from which it was derived.

$C_{36}H_{38}O_4N_4$. Calculated, N 9.5; found, N 9.6

In another experiment five 30 ml. samples of whole blood, each containing 125 mg. of isotopic glycine, were incubated as above. The isotope concentrations of the pooled hemin, directly isolated and after each of two recrystallizations, were 0.068, 0.070, and 0.072 atom per cent excess N^{15} respectively. These differences lie within the experimental error of the analytical method.

Synthesis As Function of Time of Incubation—20 ml. samples of blood from the same duck were incubated with 10, 50, and 100 mg. of isotopic glycine for 6, 12, and 24 hours. The results shown in Fig. 1 demonstrate that the rate of synthesis of heme is most rapid during the first few hours; the rate then decreases but the synthesis continues for at least 24 hours.

N^{15} Concentration of Heme As Function of Amount of Glycine Added—To study the effect of glycine concentration on the synthesis, whole blood samples (16 to 24 ml.) were incubated with 1 to 300 mg. of isotopic glycine. Glycine was added either in crystalline form or as an isotonic solution. The data in Tables I and II show that the maximum N^{15} concentration in the heme is attained by the addition of about 1 mg. of glycine per ml. of blood. The amount of glycine required to yield a maximum N^{15} concentration in the hemin will depend clearly on the amount of non-

isotopic glycine present in the blood sample. The synthetic rate does not depend on whether the glycine is added as an isotonic solution or dissolved in the blood (compare Experiments VP-33 in Tables I and II).

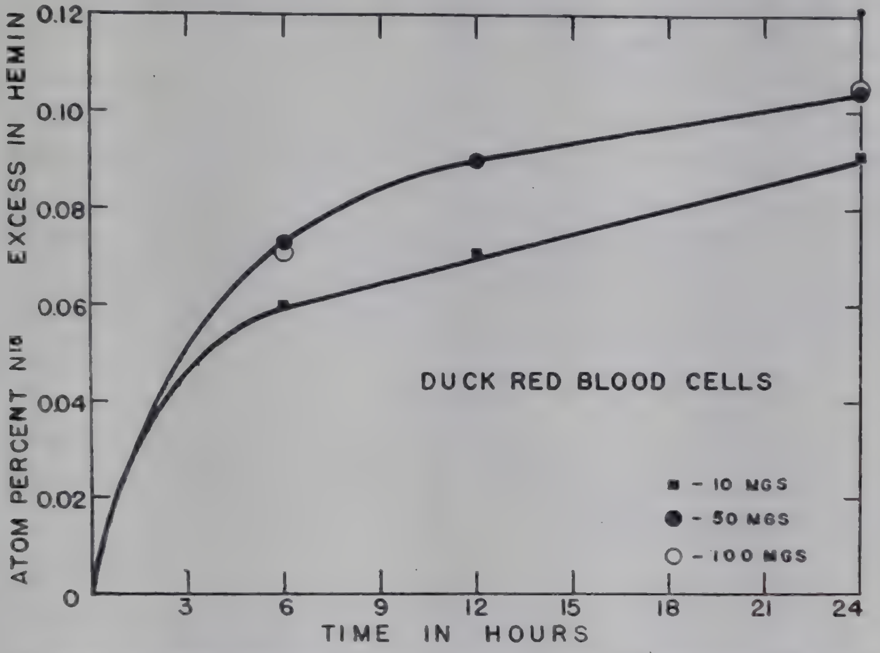

FIG. 1. Effect of concentration of glycine and time of incubation on N^{15} concentration in heme of duck erythrocytes.

TABLE I

N^{15} Concentration in Heme As Function of Amount of Glycine Added

Duck blood incubated for 24 hours with different amounts of isotopic glycine added in solid form.

Glycine added	N^{15} concentration in hemin		
	Experiment VP-28, 16 ml. blood	Experiment VP-33, 24 ml. blood	Experiment VP-36, 20 ml. blood
mg.	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
5		0.084	0.097
10	0.123	0.096	0.115
25	0.140	0.128	0.114
50	0.174	0.116	0.130
100	0.145	0.129	0.127
200	0.136	0.123	0.126
300		0.118	0.105

In one experiment, however, for some unexplained reason, the N^{15} concentration reached a maximum value on incubation with 3 mg. of glycine per ml. and then declined (see Experiment VP-28, Table I).

Influence of Temperature on Rate of Synthesis—To test the effect of temperature on the rate of synthesis of hemin one 20 ml. sample of blood was incubated with 200 mg. of glycine at 37°, while a similar sample was kept at 5° for 24 hours. The hemin of the sample kept at 37° contained 0.066 atom per cent excess N^{15}, while the hemin of the sample kept at 5° contained only 0.006 atom per cent excess N^{15}.

Influence of Storage of Blood on Synthesis of Heme—Storage of blood at 0–5° does not lead to rapid deterioration of the system involved in the synthesis of heme. 25 ml. of a freshly drawn sample of whole blood were incubated at 37° for 24 hours with 100 mg. of isotopic glycine. The hemin isolated contained 0.123 atom per cent excess N^{15}. Another sample of the same blood was kept for 48 hours in the refrigerator and then incubated with 100 mg. of isotopic glycine for 24 hours at 37°. The hemin isolated contained nearly the same isotope concentration, 0.118 atom per cent excess N^{15}.

TABLE II

N^{15} Concentration in Heme As Function of Amount of Glycine Added

Duck blood incubated for 24 hours with different amounts of isotopic glycine added as an isotonic solution.

Glycine added	Water added	Saline added	N^{15} concentration in hemin	
			Experiment VP-21, 20 ml. blood	Experiment VP-33, 24 ml. blood
mg.	*ml.*	*ml.*	*atom per cent excess*	*atom per cent excess*
1	0.04	8.64	0.046	
5	0.22	8.46		
10	0.43	8.25	0.091	0.109
25	1.09	7.59		
50	2.17	6.51	0.104	0.123
100	4.34	4.34	0.105	0.129
200	8.68			0.122

Blood from another duck was kept in the refrigerator for 1 week prior to incubation at 37° for 24 hours with 80 mg. of glycine. The hemin isolated contained 0.091 atom per cent excess N^{15}.

In another experiment samples of blood were shaken at 37° for varying lengths of time (6 to 24 hours) prior to the addition of 50 mg. of isotopic glycine. Five 20 ml. samples of blood were treated as follows: Sample 1 was incubated with 50 mg. of glycine immediately after the bleeding; Samples 2, 3, 4, and 5 were shaken at 37° for 5, 10, 24, and 24 hours respectively. After these periods of time, 50 mg. of glycine were added to each sample and the samples were incubated an additional 24 hours. In Sample 5, 50 mg. of glucose were added together with the glycine. The data shown in Table III indicate that storage at 37° impairs only slowly the synthetic mechanism.

Influence of Oxygen Tension—Hemin obtained from blood samples incubated in an atmosphere of 95 per cent O_2-5 per cent CO_2 contained the same concentration of N^{15} as hemin from blood samples incubated in air.

TABLE III

Effect on Synthesis of Heme on Incubation at 37° Prior to Addition of Isotopic Glycine

20 ml. duck blood samples were incubated with 50 mg. of isotopic glycine for 24 hours after being shaken at 37° for varying lengths of time.

Time kept at 37° prior to incubation with glycine	N^{15} concentration in hemin
hrs.	*atom per cent excess*
0	0.188
5	0.124
10	0.111
24	0.111
24*	0.106

* With 50 mg. of glucose.

TABLE IV

Synthesis of Heme As Function of O_2 Tension

Samples of blood were incubated with isotopic glycine in an atmosphere of 95 per cent O_2-5 per cent CO_2, in air, or under anaerobic conditions. The samples were made anaerobic by passing a 95 per cent N_2-5 per cent CO_2 gas mixture through the flasks for different lengths of time and the flasks were then stoppered.

Experiment No.	N^{15} concentration in hemin			
	95 per cent O_2-5 per cent CO_2	Air	Anaerobic flasks, flushed with N_2-CO_2	
			5 min.	10 min.
	atom per cent excess	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
VP-8	0.126	0.122		
VP-34		0.133		0.063
		0.134		0.043
VP-38		0.114	0.030	0.025
VP-23	0.089	0.093		

However, hemin from blood which had been incubated in an atmosphere of 95 per cent N_2-5 per cent CO_2 contained considerably less N^{15} than that isolated from samples incubated under aerobic conditions (see Table IV).

Influence of Progressive Washing of Red Blood Cells—These experiments were carried out to determine whether any constituent of the plasma is required for the synthesis of heme *in vitro*.

The blood obtained from a duck was divided into six 20 ml. portions. One sample was left unchanged. The others were centrifuged and the plasma removed. The cells from one of these samples were resuspended in a mixture of 8 ml. of 0.9 per cent saline and 3 ml. of 0.154 M phosphate buffer, pH 7.4. The cells from the others were washed once or more with equal volumes of 0.9 per cent saline and similarly suspended in phosphate buffer or in the original plasma. Each of the six samples was then incubated with 50 mg. of isotopic glycine for 24 hours in air. The results are given in Table V.

Synthesis with Cell-Free Preparations—Two 20 ml. samples of blood were frozen with solid CO_2 and allowed to thaw. Microscopic examination indicated that complete hemolysis had occurred. These preparations were incubated with 50 mg. of isotopic glycine for 24 hours at 37°. The hemin isolated contained 0.001 and 0.006 atom per cent excess N^{15}. Hemin from a control sample contained 0.114 atom per cent excess N^{15}.

TABLE V

Influence of Washing of Red Blood Cells on Synthesis of Heme

Cells from 20 ml. of blood samples were washed one or more times, resuspended either in phosphate buffer (pH 7.4) or in the original plasma, and incubated for 24 hours with 50 mg. of glycine.

Blood samples		N^{15} concentration in hemin
No. of washings	Resuspended in	
		atom per cent excess
Control (whole blood)		0.113
0	Buffer	0.103
1	"	0.093
3	"	0.084
5	"	0.081
5	Plasma	0.120

In another experiment hemin isolated from blood which had been homogenized with an equal volume of water prior to incubation contained 0.005 atom per cent excess N^{15}; hemin of the control contained 0.130 atom per cent excess N^{15}.

An acetone powder was prepared from 13 ml. of washed red blood cells in the usual manner. This powder (5 gm.) was dissolved in 0.10 M phosphate buffer, pH 7.4, and incubated with 200 mg. of isotopic glycine, 50 mg. of sodium acetate, 200 mg. of adenosine triphosphate, and 3 mg. of pyridoxal hydrochloride. The hemin isolated contained 0.004 atom per cent excess N^{15}.

Cyanide Inhibition—Six 24 ml. samples of blood from a duck were incubated with 50 mg. of N^{15}-glycine and varying amounts of cyanide for 24 hours in air. The results are shown in Table VI.

Peptide Bond Synthesis—A 25 ml. sample of blood was incubated under a 95 per cent O_2-5 per cent CO_2 atmosphere for 24 hours with 10 mg. of histidine monohydrochloride containing 69 atom per cent excess N^{15} in one of the imidazole nitrogen atoms (6). After the incubation period, 1 gm. of non-isotopic histidine was added and the mixture was dialyzed. Dialysis was continued for several days after the dialysate gave a negative test for histidine. The crude hemoglobin fraction was hydrolyzed with 20 per cent hydrochloric acid. Histidine was isolated from the hydrolysate (7) and contained 0.048 atom per cent excess N^{15}.

TABLE VI

Cyanide Inhibition

24 ml. samples of duck blood were incubated with 50 mg. of glycine for 24 hours with different concentrations of cyanide added as an isotonic solution.

Sample No.	Cyanide	N^{15} concentration in hemin
	M	*atom per cent excess*
1, 2	0	0.133, 0.134
3	10^{-2}	0.019
4	10^{-3}	0.082
5	10^{-4}	0.130
6	10^{-5}	0.137

Purine Synthesis—Four 40 ml. samples of blood were incubated in air for 24 hours with 200 mg. of isotopic (32 atom per cent excess N^{15}) glycine in each. The plasma was removed by centrifugation and the cells were washed with isotonic saline and dried by lyophilization. The purines were isolated as the copper derivatives by the method of Graff and Maculla (8) and guanine was isolated from the copper purines by isoelectric precipitation. The N^{15} concentration of the guanine was 0.012 atom per cent excess N^{15}.

DISCUSSION

In the normal mammal the heme of the mature, circulating red blood cell is not in the dynamic state and remains within the cell until the erythrocyte disintegrates (9). The appearance of labeled heme in the red blood cell of the mammal after the feeding of isotopic glycine results from the synthesis of heme in the red blood cell while it is still in the bone marrow. In the bone marrow the precursors of the mature, circulating blood cells are nucleated. As the circulating red blood cells in the duck are nucleated, they are tested for their ability to synthesize heme *in vitro*.

This was found to occur. It should be noted, however, that the nucleus is not essential, for synthesis can occur with mammalian non-nucleated but immature erythrocytes (10). The erythrocytes of the peripheral blood of the duck, which are responsible for heme synthesis *in vitro*, may be immature cells. It is known that in ducks reticulocyte counts up to 20 per cent are a normal finding. It is as yet unknown whether all the nucleated cells, or only the immature nucleated red cells, are capable of carrying out synthesis of heme *in vitro*.

The rate of synthesis is maximum initially and falls off with time. Although the synthetic capacity of the cells is unimpaired at 5°, cells stored at 37° lose some of this ability in the first few hours but only slowly thereafter. The loss of synthetic capacity of the cells may be due either to the disappearance of a substance essential for heme synthesis or to cellular maturation.

Since, as has been demonstrated, glycine nitrogen is the source of all 4 nitrogen atoms of heme (11, 12), the amount of synthesis in these *in vitro* experiments is approximately 0.4 per cent ($0.13/32 \times 100$) during the 1st day.

Even thoroughly washed cells can carry out the synthesis. Since it is known that acetic acid is required for the synthesis of heme (13), the washed cells must retain acetate or potential sources of acetate.

Anaerobiosis inhibits the reaction. Appreciable inhibition by cyanide requires concentrations of 10^{-3} M or greater. Under the conditions of the experiments described here destruction of the organization of the red cell abolishes the synthesis.

It should be noted that the red cell of the duck can incorporate histidine into the cell proteins, *i.e.* synthesize proteins. By isolation and isotope analysis of the histidine of the proteins, it is possible to estimate the fraction of the proteins formed during the 24 hour incubation. Since the isolated histidine had an isotope concentration about 1/480, *i.e.* 0.048/23.0 of that added, at least 0.21 per cent of the histidine in the protein has been incorporated during the incubation period. This is similar to the rate of porphyrin synthesis in these experiments. This suggests that globin, as well heme as, is made as required for hemoglobin synthesis.

SUMMARY

1. The synthesis of heme from glycine was demonstrated in the nucleated red blood cell of the duck *in vitro*.

2. The synthesis of peptide bonds in this *in vitro* system was also demonstrated.

3. The conditions under which heme is synthesized *in vitro* in the nucleated red blood cell of the duck are described.

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THE RELATIONSHIP OF SERINE TO PORPHYRIN SYNTHESIS*

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The specific utilization of glycine for the biosynthesis of protoporphyrin has been demonstrated (1, 2). Proline, glutamic acid, leucine, and ammonia are not directly utilized for such synthesis (2). We now find that the administration to rats of N^{15}-labeled serine or acetylglycine results in isotope concentrations of the hemin similar to those obtained following labeled glycine administration (Table I). It is probable that prior to utilization the acetylglycine is hydrolyzed to glycine. This hypothesis finds support in the experiments in which acetylglycine and glycine were incubated with duck blood (Table II). Little incorporation of labeled nitrogen was found after incubation with acetylglycine, though appreciable synthesis took place with glycine itself. It is therefore improbable that acetylglycine is an intermediate in the formation of heme. Presumably the duck erythrocytes, in contrast to the whole rat, are incapable of hydrolyzing added acetylglycine.

TABLE I

Comparison of N^{15} Concentration in Heme after Feeding Isotopic Compounds to Rats

Compound fed	N^{15} concentration	N^{15} concentration in hemin	N^{15} Concentration in hemin
		Found	Calculated*
	atom per cent excess	*atom per cent excess*	*atom per cent excess*
Glycine	32	0.442	1.37
"	32	0.451	1.41
Acetylglycine	32	0.427	1.34
"	32	0.374	1.17
DL-Serine	11.6	0.155	1.33
"	11.6	0.164	1.41

* Calculated on the basis that the compound fed contained 100 per cent excess N^{15}.

The data in Table I show that in the rat serine, as well as glycine, can serve as a precursor of heme. It is known that in the mammalian organism serine is rapidly converted to glycine with retention of the amino

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TABLE II

Comparison of N^{15} Concentration in Heme after Incubating Duck Blood with Isotopic Compounds

20 ml. of whole duck blood were incubated for 24 hours at 37° with N^{15}-labeled compounds. All the compounds were added as isotonic neutral solutions.

Experiment No.	Compound added	N^{15} concentration	N^{15} concentration in hemin	N^{15} concentration in hemin
			Found	Calculated*
		atom per cent excess	*atom per cent excess*	*atom per cent excess*
VP-19-1	Glycine (0.33 mM)	32	0.135	0.42
VP-19-2	“ (0.67 “)	32	0.199	0.62
VP-19-3	“ (1.33 “)	32	0.139	0.44
VP-19-4	DL-Serine (1.0 mM)	11.6	0.048	0.41
VP-23-1	Glycine (0.25 mM)	32	0.090	0.28
VP-23-2	DL-Serine (0.5 mM)	32	0.040	0.12
VP-23-3	NH_4Cl (0.25 mM)	32	0.010	0.03
VP-23-4	L-Aspartic acid (0.25 mM)	31.3	0.010	0.03
VP-23-6	DL-Alanine (0.5 mM)	23	0.004	0.02
VP-23-7	Acetylglycine (0.25 mM)	32	0.012	0.04

* Calculated on the basis that the compound added contained 100 per cent excess N^{15}.

TABLE III

N^{15} Concentration in Heme of Duck Blood after Incubation with Non-Isotopic Amino Acids and Labeled Glycine or Serine

Experiment No.	Isotopic compound	N^{15} concentration	Non-isotopic compound	N^{15} concentration in hemin	N^{15} concentration in hemin
				Found	Calculated*
		atom per cent excess		*atom per cent excess*	*atom per cent excess*
VP-23-1	Glycine (0.25 mM)	32		0.090	0.28
VP-23-2	“ (0.25 “)	32	DL-Serine (2.75 mM)	0.076	0.24
VP-23-3	DL-Serine (0.5 mM)	32		0.040	0.13
VP-23-4	“ (0.5 “)	32	Glycine (2.5 mM)	0.005	0.02
VP-20-1	Glycine (0.25 mM)	32	L-Serine (2 mM)	0.133	0.42
VP-20-2	“ (0.25 “)	32	L-Alanine (2 mM)	0.133	0.42
VP-20-3	DL-Serine (1.0 mM)	11.6	Glycine (1.75 mM)	0.011	0.09
VP-20-4	“ (1.0 “)	11.6	Alanine (1.6 mM)	0.023	0.20

* Calculated on the basis that the labeled compound added contained 100 per cent excess N^{15}.

group (3). Serine may therefore function as a precursor of heme by virtue of its conversion to glycine. It is also known that glycine can be converted to serine (4–6). Either of the reactions shown below could explain the experimental results.

$$\text{Serine} \rightarrow \text{glycine} \rightarrow \text{heme} \quad (1)$$
$$\text{Glycine} \rightarrow \text{serine} \rightarrow \text{heme} \quad (2)$$

In order to evaluate these possibilities, samples of duck blood were incubated with either isotopic glycine or isotopic serine. In both cases isotopic hemin resulted (Table II). However, when the isotopic serine was mixed with large amounts of non-isotopic glycine, the isotope concentration in the hemin was extremely low (Table III). On the other hand, when isotopic glycine was incubated with large amounts of non-isotopic serine, hemin of high N^{15} concentration was obtained. These results demonstrate that serine is utilized for porphyrin synthesis by virtue of its conversion to glycine (Reaction 1).

We have tested synthesis of heme with other nitrogenous compounds in duck blood. Negative results were obtained for aspartic acid, alanine, and ammonia (Table II).

EXPERIMENTAL

The N^{15}-labeled amino acids were prepared by methods previously described (3, 7).

The experimental procedures were similar to those previously described (2, 8). In incubation experiments the amino acid hydrochlorides were neutralized and added as isotonic solutions.

SUMMARY

1. Serine is utilized for the synthesis of heme by virtue of its conversion to glycine.

2. Acetylglycine as such is not utilized for synthesis of heme.

We wish to acknowledge the valuable assistance of Miss Selma Friedman and we wish to thank Mr. I. Sucher for the isotope analyses.

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ACETIC ACID METABOLISM IN TORULOPSIS UTILIS

I. THE CULTIVATION OF TORULOPSIS YEAST ON $C^{13}H_3C^{14}OOH$ AS THE SINGLE CARBON SOURCE

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During a series of experiments with labeled amino acids as single carbon substrates for the cultivation of *Torulopsis* yeast (1, 2), it was found necessary to make a thorough investigation of the intermediary metabolism of acetic acid in order to establish the main pathways of transformation of acetyl residues into amino acid structures. With this end in view, we have prepared acetic acid, labeled with C^{13} in the methyl group and C^{14} in the carboxyl, and have used this acid as a single carbon substrate for the cultivation of *Torulopsis* yeast, previously adapted to acetic acid. After growing for a certain time on this substrate, the yeast was isolated and subjected to appropriate treatments in order to separate soluble carbohydrates, fats, and proteins. The latter were then hydrolyzed and amino acids were isolated and degraded in order to localize and estimate the isotope content of different carbon atoms of the structures.

Preparation of $C^{13}H_3C^{14}OOH$—81 gm. of $KC^{13}N$, with about 5 per cent excess C^{13} (Eastman Kodak), were precipitated as zinc cyanide and the latter mixed (after drying) with an equal weight of copper oxide and burned with oxygen in a quartz tube in 2 gm. portions. The carbon dioxide was absorbed in barium hydroxide and the barium carbonate was centrifuged, washed, and dried. Yield, 197 gm. of barium carbonate with 4.5 per cent excess C^{13}.

Carbon dioxide, liberated from the carbonate by 30 per cent perchloric acid, was reduced to methanol according to the method of Nystrom, Yanko, and Brown (3). Our yield of methanol from 197 gm. of barium carbonate was 32 gm. of redistilled product, corresponding to 90 per cent. Methyl iodide was then prepared from the methanol by a modification of the method of Tolbert (4) and carbonated in a Grignard reaction with carbon dioxide containing about 5 mc. of C^{14}. Isolation of potassium acetate was carried out according to Anker (5). The dry potassium acetate (about 50 gm.) was mixed carefully with concentrated sulfuric acid in a distilling flask and the concentrated acetic acid was distilled off at 112–119°. Yield, 23 gm. of 94 per cent $C^{13}H_3C^{14}OOH$ with 3 to 4 per cent excess C^{13} in the methyl group, and about 3 mc. of C^{14} in the carboxyl group.

Experimental Conditions for Adaptation of Torulopsis Yeast to Acetic Acid—During the stages of adaptation of the yeast to acetate, we followed, in principle, the directions given by Roine (6) and Sperber (7) for small scale cultures. A few cells of a pure culture of *Torulopsis utilis* were cultivated under aerobic conditions in an ordinary wash-bottle, sterile air being passed through the culture at 30°. The medium consisted of 50 gm. of pure sucrose, 4 gm. of ammonium sulfate, 3 gm. of ammonium phosphate (secondary), 1.5 gm. of potassium sulfate, 1 gm. of magnesium sulfate, and 0.5 gm. of calcium chloride in 1000 ml. of tap water, of which 150 ml. were used during the first stage of cultivation. After the yeast had increased in wet weight to about 10 gm. (24 hours), it was transferred to a 1.2 liter Kluyver flask (see Sperber (7), Fig. 1, p. 12) containing 1000 ml. of the above medium and cultivated for 24 hours under vigorous aeration. The pH was kept at 4.0 to 4.5, usually adjusted by addition of 1 N KOH. When the wet weight had reached 80 gm., the yeast was separated from the medium by centrifuging and suspended in 2000 ml. of the same medium in a 20 liter stainless steel Kluyver flask. During all cultivation procedures, foaming was kept under control by adding, from time to time, a few drops of Turkey-red oil. The culture in the 20 liter flask was permitted to grow during 24 hours with aeration corresponding to 20 liters of air per minute. At the end of this period (72 hours from the start) the yeast had consumed 100 gm. of sucrose and had increased its wet weight to about 250 gm. The medium was then diluted with tap water to 7000 ml., 50 ml. of ethyl alcohol and 10 gm. of ammonium sulfate were added, and cultivation was continued during 8 hours with intermittent addition of ethyl alcohol, 175 gm. in total. The volume was now 8000 ml.

At this stage, 1 gm. of acetic acid (partially neutralized with ammonia), together with 15 gm. of ethyl alcohol, was added to 4200 ml. of the yeast culture. The proportion of acetic acid subsequently was gradually increased and the alcohol was decreased. At 114 hours 102 gm. of alcohol had been added and 66 gm. of acetic acid. The volume of the culture was 7300 ml. and it contained approximately 450 gm. of yeast, which metabolized 2.5 gm. of acetic acid per hour. 2500 ml. of this culture were then diluted to 15,000 ml. with tap water and 12 gm. of acetic acid and 3 gm. of alcohol were added. In subsequent additions the amount of alcohol was further reduced until only acetate was being added. At 117 hours the volume of the culture was 16,000 ml. and there were approximately 260 gm. of yeast which utilized 7.0 gm. of acetic acid per hour at a steady rate.

During this adaptation to acetate the pH was maintained at 5.1 to 6.3 and the morphological appearance of the yeast cells was observed half hourly. If the budding rate decreased below 30 per cent, the adaptation had to be repeated from the ethanol stage. The disappearance of acetic acid from the medium was followed by withdrawing 100 ml. samples of the suspension periodically, centrifuging off the yeast, distilling the cell-free solution with steam during 45 minutes, and, finally, titrating the acetic acid in the distillate.

At the 117 hour point isotopic acid (20 gm.) was added (half neutralized with ammonia) and a 100 ml. sample of the suspension was taken after 2 minutes in order to estimate *the total amount of acetic acid, labeled plus non-labeled, present in the medium.* This acid represents the starting material of the actual isotopic experiment. Further samples were taken at 60 and 120 minutes, the experiment being stopped after 180 minutes; *i.e.*, in all, after 120 hours. The yeast was then killed by adding 20 ml. of 30 per cent perchloric acid, suspended carefully in water, and centrifuged. After washing again with water, centrifuging, and drying by treatment four times with 1000 ml. portions of absolute ethanol and then three times with 400 ml. of ether, the yeast had a dry weight of 82.5 gm.

Isotope Content of Yeast and Respiratory CO_2—During the later stages of the experiment the large Kluyver flask was provided with a close fitting top with three holes, one fitted with a separatory funnel for adding substrate, one with a wide glass tube leading to the bottom of the flask, enabling samples to be withdrawn at intervals, and the third with an outlet nozzle for connection to a series of CO_2 absorption flasks containing $Ba(OH)_2$ solution. Four of the absorption flasks were coupled in series, and at intervals the flasks were exchanged against another absorption unit of the same type. Each flask contained about 1000 ml. of saturated baryta solution. In this way nine different samples of respiratory CO_2 were collected, representing the total output of CO_2 during the last 3 hours of the experiment. C^{13} and C^{14} determinations of the isolated *barium carbonate* are shown in Table I, the last columns of which give the percentage of the methyl and the carboxyl groups of the acetic acid in the medium appearing as respiratory CO_2.

The latter series of figures is based upon the isotope content of the acetic acid of the medium taken 2 minutes after addition of the isotopic acetic acid. The acetate was isolated as its silver salt by steam distillation of the sample, neutralization with KOH, evaporation to a small volume, and precipitation with silver nitrate solution. The silver acetate was burned to CO_2, which was isolated as barium carbonate and analyzed for C^{13} and C^{14}. The former is expressed in atom per cent excess, the latter in counts per minute per 15 mg. of $BaCO_3$ sample under standard conditions. All isotope measurements were made in the same way. The figures thus obtained for the isotopic acetate *actually present at 2 minutes after the start of the experiment* were 1.21 per cent C^{13} excess and 4925 c.p.m., representing

2.42 per cent C^{13} in the CH_3, and 9850 c.p.m. in COOH. With these values taken to unity, the ratio $'C^{14}:'C^{13}$, calculated for the respiratory CO_2, as shown in Table I, remained at approximately 2 during the main part of the experiment, indicating that, for each methyl group of the acetic acid metabolized to CO_2 by the yeast, two carboxyl groups are involved in decarboxylation reactions. Assuming the validity of the Krebs cycle in *Torulopsis*, this value of 2 would reflect an analogous ratio of $C^{14}:C^{13}$ in the α-carboxyl group of glutamic acid and *both* carboxyls of aspartic acid at the end of the experiment. Preliminary determination of these ratios gave the figures 1.72 and 1.78, respectively, which agreed with the ratio 1.74 for the respiratory CO_2 during the last 60 minutes of the experiment (Table I).

TABLE I

Isotope Content of Respiratory CO_2 Taken at Intervals

C^{13} in atom per cent excess, C^{14} counts per minute per 15 mg. of barium carbonate. $'C^{13}$ and $'C^{14}$ denote isotope content of respiratory CO_2 in per cent of the corresponding C^{13} content of the methyl group and C^{14} of the carboxyl group of the acetic acid present at the start of the experiment (2.42 per cent excess C^{13} and 9850 c.p.m. per 15 mg. of barium carbonate).

Time intervals	C^{13}	C^{14}	$'C^{13}$	$'C^{14}$	$'C^{13} + 'C^{14}$	$'C^{14}:'C^{13}$
min.						
0- 2	0.02	418	0.8	4.2	5.0	5
2- 7	0.17	784	6.9	7.9	14.8	
7- 22	0.24	2920	10.0	29.9	39.5	2.99
22- 42	0.45	4655	18.5	47.0	65.5	2.55
42- 65	0.52	5155	21.4	52.2	73.6	2.45
65- 95	0.62	5185	25.6	52.3	77.9	2.04
95–125	0.65	5360	27.1	54.3	81.4	2.00
125–155	0.73	5915	30.0	59.8	89.8	1.99
155–180	0.76	4990	31.4	50.6	82.2	1.62

Preliminary information about the isotope content of the samples of yeast taken at 2, 60, 120, and 180 minutes was obtained by combustion of a small amount of each of these samples to CO_2 and subsequent determination of C^{13} and C^{14}. These values, 0.060, 0.123, 0.169, 0.180, and 29, 69, 305, and 348 c.p.m., show that, even at 60 minutes, the yeast had incorporated a considerable amount of isotopic carbon from the acetic acid. In order to investigate the partition of C^{13} and C^{14} in the three main amino acid groups, the acidic, neutral, and basic fractions, the yeast was hydrolyzed with 20 per cent HCl, evaporated to dryness, and electrodialyzed according to the method of Sperber (8). From the different fractions, total carbon was determined by combustion and amino acid carboxyl liberated as CO_2 by the method of Van Slyke *et al.* (9). Table II gives the values of C^{13} and C^{14} for the total and the carboxyl C of each fraction. The total C values of the neutral fractions should be regarded with some reservation on account of their probable carbohydrate content. In the case of the basic fraction, paper chromatography showed a minor amount of ethanolamine and glucosamine.

The general picture of the incorporation of acetic acid into the three amino acid fractions, as shown in Table II, gives the impression that glutamic and aspartic acids have incorporated the isotopic carbon from acetate at a higher rate than the other amino acids. This is by no means unexpected in view of the results obtained by Lifson *et al.* (10). The decrease of the isotopic content in the acidic fraction during the last hour of the experiment might imply that part of this fraction is utilized for the synthesis of other amino acids, the members of the basic fraction in general being formed more slowly than the neutral amino acids. A feature of definite interest is, however, the high C^{14} content in the neutral and basic fraction in comparison with the corresponding values of the carboxyls from these fractions. This means that a considerable amount of the carboxyl part of the acetic acid has become incorporated into the non-carboxylic parts of some amino acid or acids, both neutral and basic. In order to locate the actual sites of C^{13} and C^{14} derived from the acetic acid in the framework of each amino acid, the 180 minute fractions, corre-

TABLE II

Isotope Content of Different Amino Acid Fractions Prepared from Four Samples of Yeast Taken at Different Stages during Experiment

C^{13} in atom per cent excess, C^{14} counts per minute per 15 mg. of barium carbonate.

Time	Carboxyl						Total					
	Acid fraction		Neutral fraction		Basic fraction		Acid fraction		Neutral fraction		Basic fraction	
	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}
min.												
2	0.00	65	0.00	16	0.00	12	0.03	47	0.02	7	0.02	24
60	0.08	524	0.02	249	0.01	102	0.16	231	0.07	54	0.08	108
120	0.15	988	0.04	464	0.04	269	0.30	380	0.14	176	0.12	156
180	0.15	902	0.09	660	0.04	314	0.28	392	0.21	244	0.17	226

sponding to about 40 gm. of protein as amino acids, were taken as starting material for isolation of individual amino acids in a pure state, each amino acid being subjected to degradation procedures in order to locate the C^{13} and C^{14} in the molecule. This work, the results of which are to be described in subsequent publications, will give some detailed information as to the function of acetic acid in the formation of individual amino acids.

SUMMARY

1. A detailed scheme is given for the cultivation of the yeast *Torulopsis utilis* on acetic acid and ammonia.

2. In a cultivation experiment, acetic acid, labeled with C^{13} in the methyl group and C^{14} in the carboxyl group, was used as a single carbon substrate for the growth of the yeast.

3. Isotope determinations of the respiratory carbon dioxide liberated during the experiment show a $'C^{14}:'C^{13}$ ratio of about 2, in comparison to the corresponding ratio of the carbon atoms of acetic acid of the substrate, taken as 1, indicating that, for *two* carboxyl groups liberated from the acetic acid as CO_2, *one* methyl group is entering the cycle of metabolic breakdown, finally appearing as respiratory CO_2.

4. In agreement with this, the C^{13} content of the yeast substance formed is relatively higher than the corresponding C^{14} content, the latter appearing mainly in the carboxyls of the amino acids formed but also to some extent in the non-carboxylic parts of some neutral and basic amino acids.

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ACETIC ACID METABOLISM IN TORULOPSIS UTILIS

II. METABOLIC CONNECTION BETWEEN ACETIC ACID AND TYROSINE AND A METHOD OF DEGRADATION OF THE PHENOLIC RING STRUCTURE IN TYROSINE

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A previous paper in this series (1) describes conditions necessary for the growth of *Torulopsis* yeast when acetic acid containing excess C^{13} in the methyl and C^{14} in the carboxyl group is fed as the sole source of carbon. This communication embodies a detailed study of the distribution of C^{13} and C^{14} in the tyrosine isolated from a hydrolysate of protein from this yeast.

The neutral fraction of the protein hydrolysate was evaporated to a small volume, the pH adjusted to 5.8, and the temperature kept at 0° for 2 days. Tyrosine crystallized out (1006 mg. of crude product) and was recrystallized from water. 580 mg. of pure amino acid were obtained from 80 gm. of dry yeast. The preparation was shown by paper chromatography to contain no other amino acids.

Degradation Procedure—In order to obtain a complete picture of the isotope distribution in tyrosine, numerical values for the C^{13} and C^{14} concentrations in the following carbon atoms must be obtained: The carboxyl, the α, and the β atoms of the side chain, and finally the atoms of positions 1, 4, 3 and 5, and 2 and 6 of the benzene ring. Positions 3 and 5, like 2 and 6, are naturally indistinguishable. The scheme presented in Fig. 1 gives the general outline of the procedure.

All measurement of isotope concentration was carried out on barium carbonate samples derived from the carbon atoms from one or several different positions of the tyrosine molecule. The isotope concentration of the barium carbonate preparations will in the following calculation be designated as C_{carboxyl}, C_α, C_β, C_1, $C_{1,4}$, $C_{1,3,5}$, etc., the suffixes indicating the original position of the carbon atoms. Two or more suffixes, *e.g.* $C_{1,3,5}$, indicate that the measured isotope concentration is the mean value of the isotope concentrations of each of the atoms in the positions indicated.

Carboxyl carbon was determined readily by treatment with ninhydrin according to the method of Van Slyke *et al.* (2), the evolved carbon dioxide being collected and analyzed as barium carbonate. The value for C_α

was obtained indirectly by conversion of the main part of the tyrosine to 4-hydroxybenzoic acid by alkali fusion. A sample of this acid, on combustion to carbon dioxide, gave the average isotope content of the ring

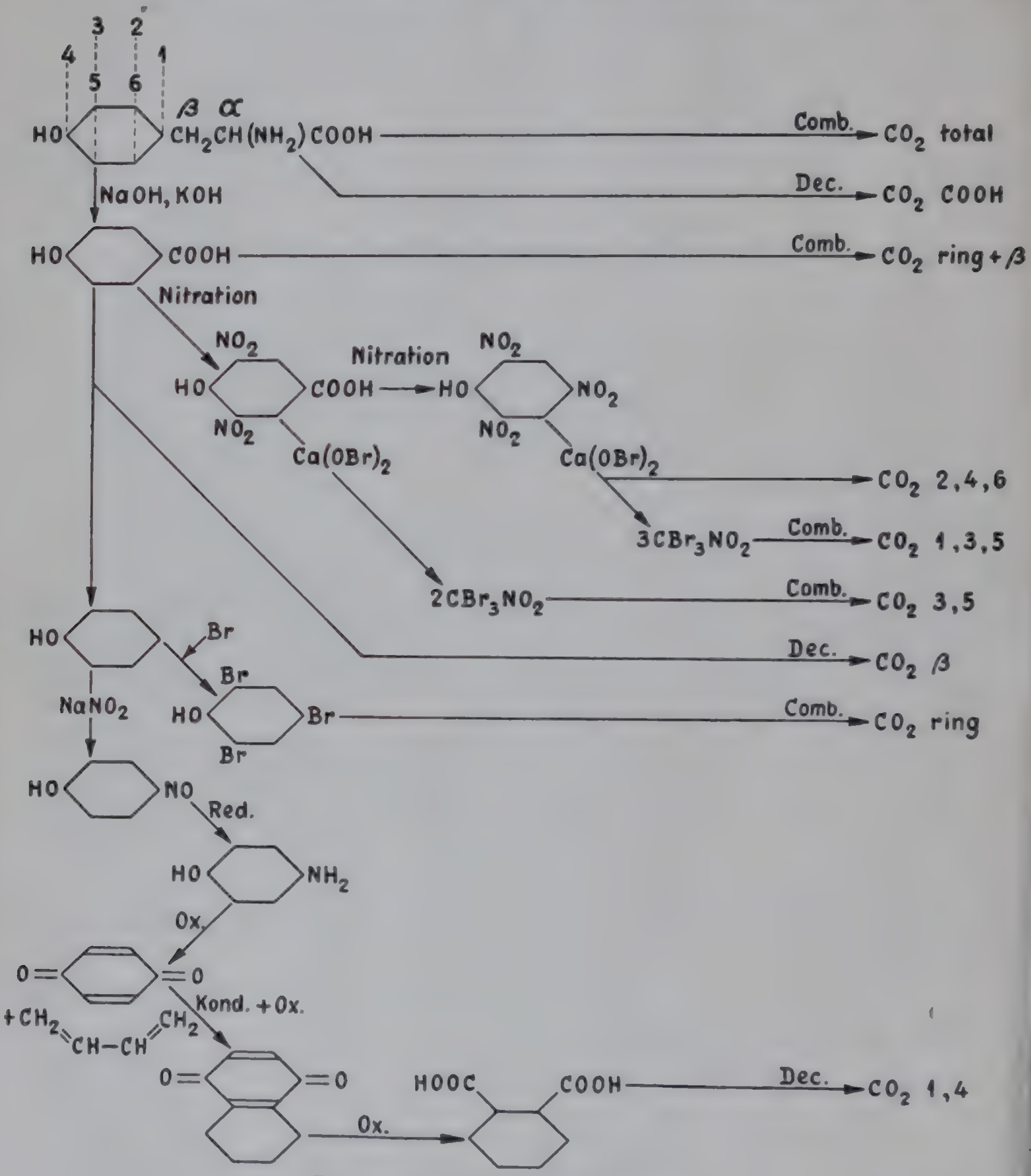

3 2
4 1
5 6
β α
HO CH2CH(NH2)COOH
Comb. CO2 total
NaOH, KOH
Dec. CO2 COOH
HO COOH
Comb. CO2 ring + β
Nitration
NO2
Nitration
NO2
HO COOH HO NO2
NO2 NO2
Ca(OBr)2
Ca(OBr)2
CO2 2,4,6
3CBr3NO2
Comb. CO2 1,3,5
2CBr3NO2
Comb. CO2 3,5
HO
Br
Dec. CO2 β
Br
NaNO2
HO Br
Comb. CO2 ring
Br
HO NO
Red.
HO NH2
Ox.
O= =O
Kond. + Ox.
+CH2=CH–CH=CH2
O= =O
HOOC COOH
Dec. CO2 1,4
Ox.

FIG. 1. Degradation of tyrosine

atoms plus C_β, which, together with the average for the whole tyrosine molecule obtained by total combustion, made it possible to calculate the value for C_α by difference, from the carboxyl figure:

$$C_\alpha = 9C_{\text{total}} - 7C_{\text{hydroxybenzoic acid}} - C_{\text{carboxyl}}$$

The carbon dioxide, evolved on heating a sample of this 4-hydroxybenzoic acid in quinoline at 250°, represented the β-carbon atom of tyrosine, while the other product of this reaction, phenol, isolated as 1,3,5-tribromophenol, on combustion to carbon dioxide gave the average of the 6-ring atoms. Nitration of a further part of the 4-hydroxybenzoic acid to 3,5-dinitro-4-hydroxybenzoic acid, followed by treatment with calcium hypobromite at 40°, caused disruption of the ring, liberating carbon atoms 3 and 5 as bromopicrin (tribromonitromethane). The latter was isolated and burned to carbon dioxide, the isotope content of which gave values for $C_{3,5}$. Further vigorous nitration, with decarboxylation of the remaining 3,5-dinitro-4-hydroxybenzoic acid, produced picric acid which, in turn, was converted to bromopicrin with calcium hypobromite. Combustion of this compound gave carbon dioxide, representing atoms 1 and 3,5. The isotope content of 1 could then be obtained from $C_1 = 3C_{1,3,5} - 2C_{3,5}$. Thus values for C_1 and $C_{3,5}$ were obtained, while the difference between the carbon of the phenol and $C_{1,3,5}$ gave $C_{2,4,6}$. In order to distinguish between C_4 and $C_{2,6}$, the main part of the phenol obtained by decarboxylation of 4-hydroxybenzoic acid was converted to 4-nitrosophenol by treatment with nitrous acid. This, on reduction to 4-aminophenol, followed by oxidation, gave *p*-benzoquinone, which was condensed with 1,3-butadiene to dihydronaphthoquinone. Oxidation of the latter by the method of Fieser (3) gave naphthoquinone, which could be oxidized further with permanganate to phthalic acid. The phthalic acid, on decarboxylation, liberated one carboxyl as carbon dioxide, the isotope content of which corresponded to $C_{1,4}$ in the original phenolic ring. (In view of the symmetry of *p*-benzoquinone, positions 1 and 4 are indistinguishable by this degradative procedure, the isotope determinations on the carbon dioxide from phthalic acid giving the mean of atoms 1 and 4.) However, since the value of C_1 was known from the previous degradation, it was possible to calculate C_4 in the following way: $C_4 = 2C_{1,4} - C_1$. Also, the isotope content of the 2 equivalent atoms, 2 and 6, follows from $C_{2,6} = 3C_{\text{phenol}} - C_{3,5} - C_{1,4}$.

EXPERIMENTAL

4-Hydroxybenzoic Acid—475 mg. of tyrosine were added in small portions to a molten mixture of 2 gm. of potassium hydroxide and 2 gm. of sodium hydroxide and kept at 255–265° for 12 minutes, at which time gas evolution had ceased. The reaction vessel was a small silver crucible, heating being carried out with an ordinary Bunsen flame controlled manually. The mixture was cooled and dissolved in a minimum quantity of water, acidified with 50 per cent sulfuric acid and extracted five times with ether. The dried ether extracts were evaporated and the residue was dissolved in 4 ml. of 1 N sodium hydroxide. A small amount of norit was added; the solution was then filtered and acidified to pH 2 with sulfuric acid. After a short time, 4-hydroxybenzoic acid separated as colorless crystals. The product, after filtration and washing with ice water, was redissolved in alkali, neutralized, and cooled. The crystals, after they had been washed and dried *in vacuo*, melted at 211°, and there was no depression when mixed with authentic 4-hydroxybenzoic acid. Yield; 197 mg.

15 mg. of the acid were used for analysis and combustion to CO_2. The remaining 182 mg. were dissolved in ether and carefully washed with ether into a solution of 364 mg. of recrystallized 4-hydroxybenzoic acid. The dilution of the preparation, after evaporation of the ether, should be 1:3, which was confirmed by C^{13} and C^{14} determination both before and after dilution. The per cent of C^{13} before and after dilution was 0.183 ± 0.005 and 0.065 ± 0.002, respectively, the corresponding C^{14} figures being 129 ± 5 and 43 ± 2 c.p.m. per 15 mg. of barium carbonate, which give dilutions of 2.86 ± 0.10 and 3.09 ± 0.14. All the following degradations were carried out on the diluted 4-hydroxybenzoic acid, and the isotope contents of the degradation products compared with each other, for which the dilution value of 3.0 was accepted.

3,5-Dinitro-4-hydroxybenzoic Acid—The method of Morgenstern (4) was followed with some modifications. In a small test-tube 50 mg. of the above 4-hydroxybenzoic acid (diluted 1:3) were dissolved at room temperature in 0.27 ml. of concentrated sulfuric acid. The solution was cooled in ice water and an ice-cold mixture of 0.17 ml. of concentrated sulfuric acid and 0.17 ml. of nitric acid, density 1.4, was added. After a short time, the nitration was complete and the reaction mixture was kept at 0° for 2 hours. The crystals were filtered off on a small sintered glass filter, washed with small amounts of ice water, sucked dry, washed with hot benzene, and finally recrystallized from 2 ml. of 70 per cent ethanol. The dry acid melted at 244° and on admixture with an authentic sample melted at 243–244°. Yield, 33 mg.

Picric Acid—110 mg. of 4-hydroxybenzoic acid were converted to 3,5-dinitro-4-hydroxybenzoic acid as before, except that the dinitro acid which crystallized from the acid mixture was not isolated but subjected to further nitration. To the reaction mixture, originally containing 0.90 ml. of concentrated sulfuric acid and 0.35 ml. of nitric acid, density 1.4, an additional amount of 0.35 ml. of nitric acid, density 1.4, was added and the mixture slowly heated on the water bath to 100°. After some foaming (carbon dioxide evolution), the solution became a clear, pale yellow. Heating was continued for 2 hours, when the solution was cooled in ice water, diluted with 3 volumes of ice water, and kept at 0° for 2 hours. The crystals were filtered off, washed with a minimum quantity of ice water, and twice recrystallized from 4 ml. of water. Yield, 56 mg. of picric acid; m.p. 122°; unchanged on mixing with an authentic sample.

Bromopicrin (*Tribromonitromethane*)—The degradations of 3,5-dinitro-4-hydroxybenzoic acid and picric acid were carried out under identical conditions. In two separate experiments, 32.2 mg. of the dinitro acid and 28.4 mg. of picric acid were dissolved in 1 ml. of water and 10 mg. of calcium hydroxide. 10 gm. of a paste made up from 7.5 gm. of calcium hydroxide, 30 ml. of water at 0°, and 2.5 ml. of bromine were added. After mixing and heating to 40°, the yellow color of the mixture gradually faded and a strong smell of bromopicrin was apparent. The reaction mixture was distilled and 3 ml. of the distillate were collected in a 10 ml. centrifuge tube with a conical tip. The small amount of oily bromopicrin was centrifuged down into the tip of the tube, the supernatant fluid was sucked off, and the oil droplet washed twice by centrifuging with distilled water. Finally, the bromopicrin droplet was transferred by the aid of a micro pipette to a platinum boat and burned in oxygen over a platinum catalyst to carbon dioxide, which was trapped and isolated as barium carbonate. The yield of bromopicrin from 3,5-dinitro-4-hydroxybenzoic acid was 62.7 mg., and from picric acid 67.5 mg. Since the amount of bromopicrin isolated was so small, its purity could not be ascertained, but it melted at about 5°, the recorded melting point being 10°. A control experiment with larger amounts of unlabeled acids gave directly a product with a boiling range of 122–127° (recorded b.p. 127°). The bromine used in the reaction was free from brominated hydrocarbons.

In a separate experiment, 27.8 mg. of picric acid were degraded in a similar way with carbonate-free barium hypobromite, a solution of which was filtered directly into the reaction flask. The bromopicrin was treated as above and the carbon dioxide evolved trapped in barium hydroxide solution. This carbon dioxide represented carbon atoms 2, 4, 6 in the original 4-hydroxybenzoic acid. 82.6 mg. of barium carbonate were obtained, whereas a control experiment run without any nitro compound gave 7.0 mg. Thus 75.6 mg. were obtained from the picric acid and corresponded to a yield of 97 per cent.

p-Benzoquinone—361 mg. of 4-hydroxybenzoic acid were dissolved in 3 ml. of pure, redistilled quinoline; 100 mg. of pure copper powder were added and the mixture was heated for 50 minutes in a metal bath maintained at 255–260°. The 15 ml. reaction flask was connected to a 20 cm. air condenser, the end of which was connected to a centrifuge tube containing filtered, saturated, barium hydroxide solution. Throughout the reaction period, dry, carbon dioxide-free nitrogen was passed through the mixture in order to sweep out the carbon dioxide liberated. The barium carbonate isolated (480 mg., 94 per cent yield) was analyzed for isotopes and considered to be identical with the β atom of tyrosine.

The phenol remaining in the quinoline solution after completion of the decarboxylation was isolated by adding 10 ml. of 30 per cent sulfuric acid to the reaction mixture, followed by careful extraction with ether. The ether extract was shaken six times with 1 ml. portions of 1 N sodium hydroxide solution and the combined alkaline extracts decolorized with a small amount of norit. After filtration and neutralization with 30 per cent sulfuric acid, a small amount of this solution was treated with bromine water. The tribromophenol was sucked off, dried, and burned to CO_2, trapped as barium carbonate, in order to obtain the isotope value for the sum of the 6 carbon atoms of the ring. The rest of the phenol solution (about 200 mg.) was transferred to a 25 ml. flask, 90 mg. of solid sodium hydroxide and 190 mg. of dry sodium nitrite were added, and the solution was cooled in a freezing mixture to −5°. The nitrosation procedure followed in general the directions given by Bridge (5). 1.68 ml. of a sulfuric acid solution (1 volume of concentrated acid and 5 volumes of water) were added carefully during 5 minutes with cooling and shaking. After addition of the sulfuric acid, the mixture was kept at 0° for 2 hours, during which time most of the 4-nitrosophenol crystallized out as needles.

Without separating the crystals from the mother liquor, the reaction mixture was made alkaline by addition of 3 ml. of ammonia (density 0.91), and hydrogen sulfide was passed in for half an hour. After this time, the solution, which now contained 4-aminophenol and a small amount of sulfur, was made neutral by addition of concentrated sulfuric acid, the liberated hydrogen sulfide being swept out by a stream of nitrogen.

To the solution, now pale yellow and somewhat turbid because of the presence of small amounts of sulfur, 0.3 ml. of concentrated sulfuric acid was added, immediately followed by 50 mg. of vanadium pentoxide and 3 gm. of lead dioxide (PbO_2). The mixture was shaken vigorously for 10 minutes. After the first few minutes, the color became dark violet and the temperature rose to about 40°. Later, this color disappeared and, after 10 minutes, the reaction mixture was subjected to steam distillation at 40 mm. pressure, the quinone distilling over into a receiver cooled in a freezing mixture. When the vapors were no longer colored, the condensers were disconnected from the distilling flask and rinsed with small amounts of ether. The total distillate was extracted with ether until the aqueous layer was colorless; the combined extracts were then dried over sodium sulfate and evaporated very carefully to dryness at 40°. The quinone was dissolved immediately in pure pentane at 35° and the solution filtered from a little black, insoluble material and evaporated to 5 ml. After cooling with solid carbon dioxide and acetone, benzoquinone crystallized as

TABLE I

Determined and Calculated Isotope Content of Degradation Products of Tyrosine, and Isotope Content of Individual Atoms of Tyrosine Structure

C_{total} denotes total carbon as barium carbonate by combustion; C_{COOH} carboxyl carbon. All the measurements were made on duplicate samples. C^{13} is given in atom per cent excess, C^{14} in counts per minute per 15 mg. of $BaCO_3$.

Series	Compounds investigated	Position of C atoms in tyrosine	Isotope values				Calculations, based on series
			Determined		Calculated		
			C^{13}	C^{14}	C^{13}	C^{14}	
A	Tyrosine, C_{total}	All atoms	0.190 ±0.005 0.201 ±0.005	153 ±6			
B	" C_{COOH}	COOH	0.090 ±0.005	458 ±8			
C	4-Hydroxybenzoic acid, C_{total}	Ring, β	0.185 ±0.005	129 ±6			
D	4-Hydroxybenzoic acid, C_{COOH}	β	0.255 ±0.005	12 ±5			
E		α			0.27 ±0.05	17 ±20	A, B, C
F	Benzene ring (Tribromophenol, C_{total})	1, 2, 3, 4, 5, 6	0.190 ±0.005	146 ±7			
G	Benzene ring	1, 2, 3, 4, 5, 6			0.18 ±0.02	152 ±10	C, D
H	Bromopicrin, C_{total}, from picric acid	1, 3, 5	0.191 ±0.01	91 ±4			
I		1, 3, 5			0.17 ±0.02	65 ±15	F, L
J	Bromopicrin, C_{total}, from 3,5-dinitro-4-hydroxybenzoic acid	3, 5	0.135 ±0.005	122 ±7			
K		1			0.27 ±0.03	26 ±18	H, I, J
L	CO_2 from bromopicrin-split H	2, 4, 6	0.21 ±0.01	233 ±15			
M	Phthalic acid, C_{total}			88 ±6			
N	" " C_{COOH}	1, 4	0.140 ±0.015	268 ±8			
O		1, 4				266 ±40	F, G, M
P		4			0.05 ±0.02	506 ±20	K, N
		4				482 ±40	F, G, M, K
Q		2, 6			0.25 ±0.02	60 ±15	F, G, J, N
R		2, 6			0.29 ±0.03	99 ±40	L, P

yellow needles, which were filtered off and washed with cold pentane. An additional amount could be obtained from the mother liquor. Total 47.3 mg.; m.p. 113°. The product sublimed without residue.

1,4-Naphthoquinone—The preparation of naphthoquinone was carried out by the method of Fieser (3). 44.5 mg. of *p*-benzoquinone and 52 μl. of pure, redistilled 1,3-butadiene in 0.27 ml. of glacial acetic acid were kept at room temperature for 44 hours in a thick walled 1 ml. flask fitted with a carefully ground glass stopper. 0.25 ml. of a solution of 4 gm. of sodium dichromate and 0.2 ml. of concentrated sulfuric acid in 2.5 ml. of water were mixed with 0.1 ml. of acetic acid and added to the reaction mixture at 65°. After 30 minutes an additional amount (0.1 ml.) of the oxidation mixture was added and the contents of the flask shaken occasionally. At the end of 50 minutes, the flask was cooled and the contents diluted with 3 ml. of ice water and held at 0° for 10 minutes. The pale yellow naphthoquinone was filtered off, washed with water until the washings were colorless, dried *in vacuo* at room temperature, and weighed. Yield, 46.1 mg.; m.p. 121°; mixed m.p. 122°.

TABLE II

Comparison between Isotope Content of Acetic Acid Used As Single Carbon Substrate for Growth of Torulopsis Yeast and of Individual Atoms of Tyrosine Isolated from Yeast Proteins

[C^{13} and C^{14} values are given in the same units as in Table I. The figures for separate atoms of tyrosine are mean values compiled from the same table.

		C^{13}	C^{14}
Acetic acid of substrate	COOH		9850 ± 50
	CH_3	2.42 ± 0.02	
Separate carbon atoms of tyrosine	COOH	0.090 ± 0.005	458 ± 10
	α	0.27 ± 0.05	8 ± 8
	β	0.25 ± 0.01	12 ± 5
	1	0.27 ± 0.03	30 ± 20
	2, 6	0.26 ± 0.03	70 ± 20
	3, 5	0.135 ± 0.01	122 ± 7
	4	0.05	500 ± 50

Phthalic Acid—5.2 gm. of potassium permanganate and 1.5 ml. of concentrated sulfuric acid were dissolved in 100 ml. of water. To 4.0 ml. of this solution, 43.8 mg. of naphthoquinone were added and the reaction mixture shaken vigorously until the permanganate color had almost disappeared (about 12 minutes). The mixture was extracted continuously for 2 hours with ether. The extract was evaporated to small volume and transferred with careful washing to a 10 ml. centrifuge tube, and the evaporation was completed in this tube. The crystalline residue was washed twice with 2 ml. of chloroform, twice with pentane, and then dried at 80° for 20 minutes. Yield, 35.5 mg.; m.p. 195–200° with decomposition (recorded m.p. 191–209°); m.p. of anhydride 129° (recorded m.p. 130.5°). The purity of the preparations of phthalic acid from naphthoquinone was checked by model preparations involving larger amounts of substance.

Decarboxylation of phthalic acid was carried out by heating 30.8 mg. of the acid, dissolved in 1.5 ml. of quinoline, with 30 mg. of copper powder for 1 hour at 255°, while a slow current of carbon dioxide-free nitrogen was passed through the reaction flask to sweep out the liberated carbon dioxide. 27.8 mg. of barium carbonate were obtained from the evolved carbon dioxide by trapping the latter in barium hydroxide solution. Determination of the isotope content of the carbonate gave the values for $C_{1,4}$.

As a control of $C_{1,4}$, a small sample of the phthalic acid (4.3 mg.) was burned to carbon dioxide, which was isolated as barium carbonate. From the isotope content of the latter, $C_{2,4}$ could be calculated from the formula, $C_{1,4} = 8C_{\text{phthalic acid}} - 3C_{\text{phenol}}$.

The results of isotope determinations of the different carbon atoms of tyrosine are shown in Tables I and II.

DISCUSSION

Before considering the question of the biochemical significance of the results obtained from the degradation of tyrosine, a critical examination of the methods is justified. The weakest point, the deduction of C_1, is based on the difference between the two degradations giving rise to bromopicrin, the completeness of which may be questioned. Bromopicrin, obtained from 3,5-dinitro-4-hydroxybenzoic acid, originates from atoms 3 and 5 and, since these 2 carbon atoms are indistinguishable, an incomplete degradation here is unimportant. In the case of picric acid, however, any differences in the proportion of atoms 1, 3, and 5 converted to bromopicrin would be reflected in the values for $C_{1,3,5}$ obtained by analysis of a sample of this bromopicrin. On the other hand, previous investigators of this reaction have obtained yields of 95 to 97 per cent bromopicrin from picric acid (6) and this has been confirmed in our own experiments. An alternative method of deducing values for $C_{1,3,5}$ involves the figure obtained for $C_{2,4,6}$ from the carbon dioxide evolved during the fission of picric acid with barium hypobromite, the following equation being employed: $C_{1,3,5} = 2C_{\text{phenol}} - C_{2,4,6}$.

In this way the C^{13} content of $C_{1,3,5}$ was found to be 0.16 atom per cent excess, as compared with the value 0.191 obtained from the bromopicrin produced from picric acid. From these figures, the C^{13} content of C_1 was calculated to lie between the limits 0.24 and 0.30, the mean value 0.27 ± 0.03 being accepted. In a similar way, the C^{14} content of C_1 could be placed between the limits of 10 and 50 c.p.m. per 15 mg. of barium carbonate, expressed as the mean 30 ± 20.

If larger amounts of material had been available, reduction of 4-hydroxybenzoic acid to 4-hydroxybenzyl alcohol, then to *p*-cresol, might have been tried in an attempt to calculate C_1 directly. *p*-Cresol, on oxidation by the method of Anker (7), would give acetic acid, the carboxyl of which would be identical with carbon atom 1 of tyrosine and could be isolated by one of several well known degradation procedures.

The uncertainty attached to C_1 is naturally reflected in C_4, although in our case the very high C^{14} content of the latter must be regarded as significant. The corresponding C^{13} figures vary between 0 and 0.05, depending on the figures ascribed to C_1. In any case, it could be regarded with safety as "low" and thus the ratio $C^{14}:C^{13}$ for C_4 is analogous to that for C_{carboxyl}.

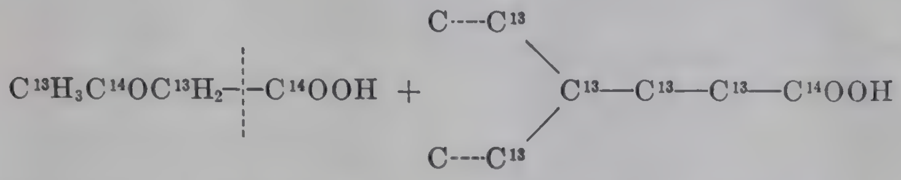
The isotope concentration in positions 2 and 6 can be calculated in two ways from the equations

$$C_{2,6} = 3C_{\text{phenol}} - C_{3,5} - C_{1,4}$$
$$C_{2,6} = \tfrac{1}{2}[3C_{2,4,6} - C_4]$$

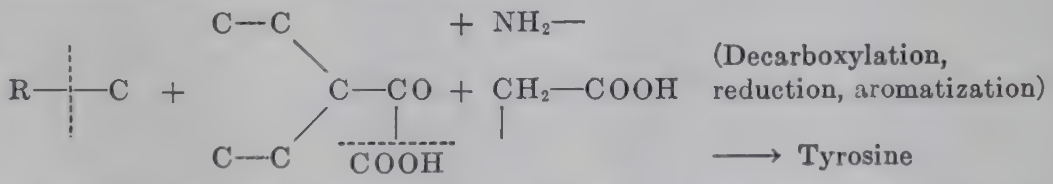
The first is regarded as more accurate, the second being influenced by the errors in C_4. The values of 0.26 ± 0.03 for C^{13} and 70 ± 20 for C^{14} might be regarded as fairly reliable.

Table II gives the figures finally accepted for the isotope content of acetic acid used and the several carbon atoms of tyrosine isolated, the errors included being the expected maximum spread and not computed mean errors, which would appear smaller than the figures given.

From the data given in Table II, there appears to be fairly strong evidence that both atom 4 and the carboxyl carbon of tyrosine are derived by more or less direct routes from the carboxyl group of administered acetic acid. The slight excess of C^{13} present in these 2 atoms would arise through recycling of acetate in oxidative breakdown processes, whereby a certain amount of the methyl moiety might be expected to appear in the carboxyl of newly formed acetate. The atoms α, β, 1, 2, and 6, with their rather uniform C^{13} content, seem to be derived from the methyl group of acetate. The "mixed" isotope content of atoms 3 and 5 makes the origin of these 2 atoms uncertain, especially since the C^{13} value of $C_{3,5}$ ought to be higher than 0.135 atom per cent excess if either atom 3 *or* 5 had come from the methyl group of an acetate molecule, the carboxyl of which would make up atom 4. As a hypothesis, we propose that atom 4 is derived either directly from carbon dioxide or formate or possibly from acetate via acetoacetate, the carboxyl of the latter making up atom 4 and being followed by ketonic fission. All these possibilities imply that the formation of the ring is connected with the joining up of a carbon atom with two ends of a branched structure which, except for its terminal atom, has been derived mainly from the methyl moiety of acetate.


$C^{13}H_3C^{14}OC^{13}H_2$–¦–$C^{14}OOH$ +
C---C^{13}
C^{13}—C^{13}—C^{13}—$C^{14}OOH$
C---C^{13}

The sequence of five rather uniformly labeled C^{13} atoms, α, β, 1, 2, and 6, must have been derived from acetate by a series of decarboxylations, perhaps more analogous to the formation of α-ketoglutarate from oxalacetate and acetate than, for instance, head to tail condensation of acetyl residues. Thus, the formation of the benzene ring system of tyrosine does *not* seem to be a special case of carbon chain formation, as is the production of fatty acids and steroids (*cf.* Bloch (8)), followed by cyclization, since then an alternating occurrence of C^{13} and C^{14} would have been found in the molecule. It seems possible that the main part of the tyrosine structure may have been derived from an α-keto acid and glycine or an α-keto acid and acetate, followed by amination of a double bond; *e.g.*, as in the formation of aspartate from fumarate.



Finally, we would like to point out that these results and their interpretation may apply only to *Torulopsis* yeast or, at least, to the lower fungi. The experiments would have to be repeated on a variety of biological materials before any general conclusions could be drawn.

Work is in progress at present on a similar degradation of phenylalanine with the object of correlating the metabolism of phenylalanine with tyrosine.

SUMMARY

A method has been developed which enables the following carbon atoms of tyrosine to be isolated in the form of carbon dioxide: the carboxyl, β, 3 and 5, 1 and 3 and 5, 2 and 4 and 6, and, finally, 1 and 4. Tyrosine obtained from a protein hydrolysate from *Torulopsis* yeast, grown on labeled acetic acid as a single carbon source, was degraded by this method and the isotope content of each carbon atom of the tyrosine determined.

The carboxyl group of administered acetic acid appears as carboxyl and the ring atom 4 of tyrosine, while the methyl group contributes mainly to α, β, 1, 2, and 6 atoms. The origin of atoms 3 and 5 is uncertain. The mechanism of tyrosine synthesis is discussed.

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COMBINATION OF WOOL PROTEIN WITH CATIONS AND HYDROXYL IONS*

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Earlier work with two proteins, wool and egg albumin, and over forty different strong acids (13, 15, 19, 20) has shown that their titration curves differ widely (up to 2.5 units) with respect to the pH axis. This effect was shown to arise from combination of proteins with anions as well as with hydrogen ions; the differences between the positions of the respective titration curves permit calculation of the relative affinities of the anions for the protein (14, 19). The relative affinities may also be deduced from the effectiveness of the anions in catalyzing hydrolysis of the amide and peptide bonds of the protein (16). Anions of moderate or high affinity, when combined on the primary or secondary amides, catalyze their hydrolysis; the catalytic effect is approximately proportional to their affinity for the protein as calculated from the titration curve.

The concept of anion binding by proteins has now been amply confirmed by work in many other laboratories (3, 4, 6, 8, 9, 23–25) by dialysis equilibrium and other methods (9) which do not depend on the interpretation of titration phenomena. The effect of added salt on the titration curve of wool is, in part, another manifestation of the combination with anions (17). Since the effect of salt on the acid titration is exactly paralleled by its effect on titration with bases, this particular protein must bind cations also, although evidence has been presented which suggests that two other proteins, serum albumin (9) and aldolase (24), may bind anions only.

If wool binds cations, the titration curves obtained with different strong bases should also differ from one another. The present investigation determines the extent to which this inference is sustained, and explores the relation of the affinity of cations for this protein to the size or shape of the ions tested.

Accurate measurements with bases are more difficult to make than with acids. This is especially true when one must use very dilute solutions (down to 10^{-5} M) and in the absence of the buffering capacity of dissolved protein. This results in extreme susceptibility to contamination by atmospheric CO_2, and to effects due to dissolving of or adsorption by glass. The following factors also limit the accuracy and possible range of the measurements with base, and are dealt with later. (*a*) Measurements of pH values with the glass electrode in the alkaline range are subject to several sources of error which must be minimized or calibrated. (*b*) Wool, like other sulfur-containing proteins, is sensitive to even very dilute alkali unless exposed for short intervals (consistent with attainment of diffusion equilibrium), at the lowest temperatures possible. (*c*) The choice of univalent bases is limited, compared with the wide choice of strong acids previously investigated. Few inorganic ions are available (Li^+, K^+, Cs^+, and Rb^+, since Na^+ must be omitted because of its effect on the glass electrode). Besides these, there are only the quaternary ammonium compounds. No attempt has been made, therefore, to calculate affinity constants, or to study the stoichiometry of the reactions.

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* Brief accounts of these measurements were reported at the meetings of the American Society of Biological Chemists on April 19, 1949, at Detroit, and at a meeting of the Washington Chemical Society on May 14 at College Park, Maryland.

EXPERIMENTAL

Except as specifically noted, the procedures employed in the purification of the wool, determination of the alkali equivalence of its ash, and measurement of the base bound were as previously described (17, 18).

Wool[1]—The ash content was 0.25 per cent. A correction of 0.042 m.eq. per dry gm., the alkali equivalence of the cationic ash as determined by electrodialysis (11), was added to the measured amount of base bound. Corrected measurements of the combination with hydrochloric acid agreed within the experimental error with earlier results (17). All results refer to dry weight of wool.

Bases—The bases used were commercial products of c.p. grade, except as described below, and were free of carbonate. Tetraalkylammonium bromides or iodides were converted into hydroxides by treating their solutions with excess silver oxide in the cold. Quaternary ammonium salts which were not available commercially were synthesized from alkyl bromides and trimethylamine by the method of Scott and Tartar (10). The molecular weights of the salts, used as a measure of their purity, differed from the theoretical values by less than a few tenths per cent, except for the octyltrimethylammonium bromide, which was 2 per cent high.

Measurement of Base Combined—The method of measurement has been described earlier (17). After the initial equilibration period, the alkaline solution withdrawn for analysis was replaced in some cases with distilled water. During a second period for equilibration the wool invariably gave up base to the solution, and a second titration curve was obtained whose coincidence with the first was proof that equilibrium had been attained.

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[1] Thanks are due to Dr. Milton Harris of the Harris Research Laboratories, Washington, D. C., for providing this material.

When the two curves did not coincide, as with tetradecyltrimethylammonium hydroxide, they set narrow limits to the range within which the true equilibrium curve must fall.

Measurements made at high base concentrations were corrected for the decomposition of disulfide bonds (18). The correction for the amount of protein dissolved was less than 2 per cent of the base bound in the worst cases (long exposure to concentrated base) and was usually negligible. The extent of decomposition of the wool depended on the concentration of base and the time of exposure, and was independent of the identity of the base, with one well marked exception: tetrabutylammonium hydroxide caused less than half as much decomposition as any other base. There was no evidence of catalysis of hydrolysis by cations, but the highest amounts bound represented a smaller fraction of the maximum than was required for catalysis by anions (16).

Measurement of pH—In the present investigation it was necessary to make pH measurements corresponding to base concentrations as high, on occasion, as 0.06 M. Since the glass electrode in alkaline solutions is subject to substantial errors, and these errors depend, in part, on the base employed, an empirical calibration procedure was resorted to for *each base used.* This procedure proved satisfactory, because the deviations of the behavior of a glass electrode from that of an ideal hydrogen electrode are very much smaller and more reproducible at 0°, in the absence of salts, than at room temperature when even small amounts of alkali salts are present. The maximum difference between indicated pH (calculated from the e.m.f. as if a hydrogen electrode were used) and true pH never exceeded about 0.2 unit, and this maximum correction was applied only at values well above pH 13. All the corrections determined could be taken into account by reading off pH values from a *linear* plot of e.m.f. increments against pH values calculated from the expression $-\log f_{\text{BOH}}C_{\text{BOH}}$ corresponding to a series of dilutions of each base (Fig. 1). This procedure, although resting on a somewhat more secure foundation than in earlier work, is essentially similar to the one previously used (18).

The calculation of pH values required for the calibration depends on values of f_{BOH} which have been taken from the literature (2), or calculated from osmotic coefficients (5). In the absence of earlier data, f_{BOH} was calculated by means of the Debye-Hückel limiting law, since, in dilute solutions, hydroxylic compounds of large cations will probably show even smaller deviations from ideality than are given by this relation.[2] Resulting uncertainties in pH will not exceed 0.03 unit in the most concentrated range, and should be negligible below pH 13.

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[2] Personal communication from Professor G. Scatchard.

No standards corresponding to concentrations of base much below 10^{-3} M were used. Such dilute unbuffered solutions of bases are too susceptible to contamination (by CO_2, dissolved glass, etc.) to be considered pH standards at all. The validity of the pH values of the most dilute solutions, read from the straight line established by borate buffer and by points between pH 12 and 13.6, is confirmed by indicator readings, and by the observation (7) that the difference in potential of a glass electrode and hydrogen electrode is constant in unbuffered solutions between pH 6.0 and 8.5.

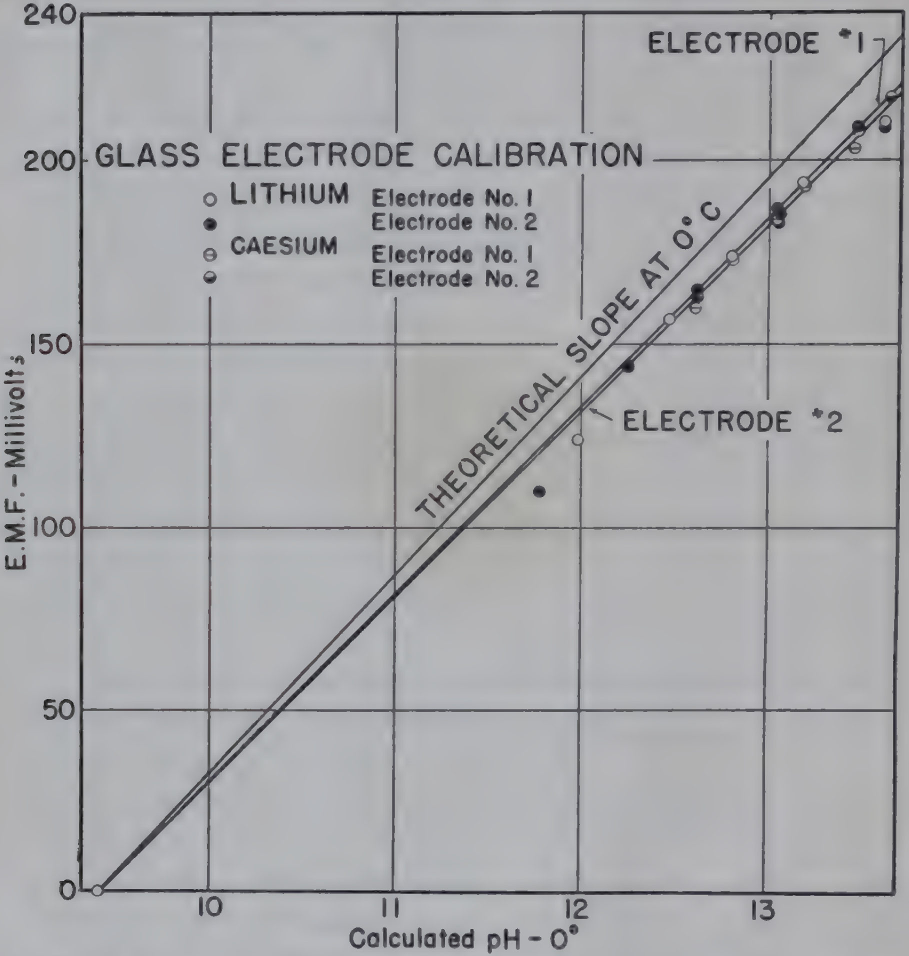

FIG. 1. Glass electrode calibrations for lithium hydroxide and cesium hydroxide. The e.m.f. increments were measured from a standard 0.05 M borate buffer, (pH 9.39 at 0°) with two different glass electrodes. The calibration curves for the two bases are indistinguishable.

RESULTS AND DISCUSSION

Since differences in the position of the base titration curves with respect to the pH axis have been found of comparable magnitude to those previously demonstrated on the acid side, it appears that large differences in the affinity of cations for wool protein parallel the affinity differences which exist for anions.

The results of a representative pair of experiments illustrating the shift in position of the curve are shown in Fig. 2. This figure also shows a number of features common to all the other experiments, as indicated in the legend. The corrections applied to the KOH data for disulfide decomposition are very small, since the protein was exposed to the solutions of base for only 3 days. At pH values below 13, the corrections may be neglected. With decyltrimethylammonium hydroxide, the wool was exposed for 17 days, and much larger amounts of decomposition resulted, noticeable at pH values as low as 12; however, the experimentally determined corrections result, when applied, in a smooth set of data. Some of the points (those which are tagged) represent measurements made after an additional 13 days immersion in solutions diluted with water after the initial period. These points, corrected for additional protein decomposition, are wholly con-

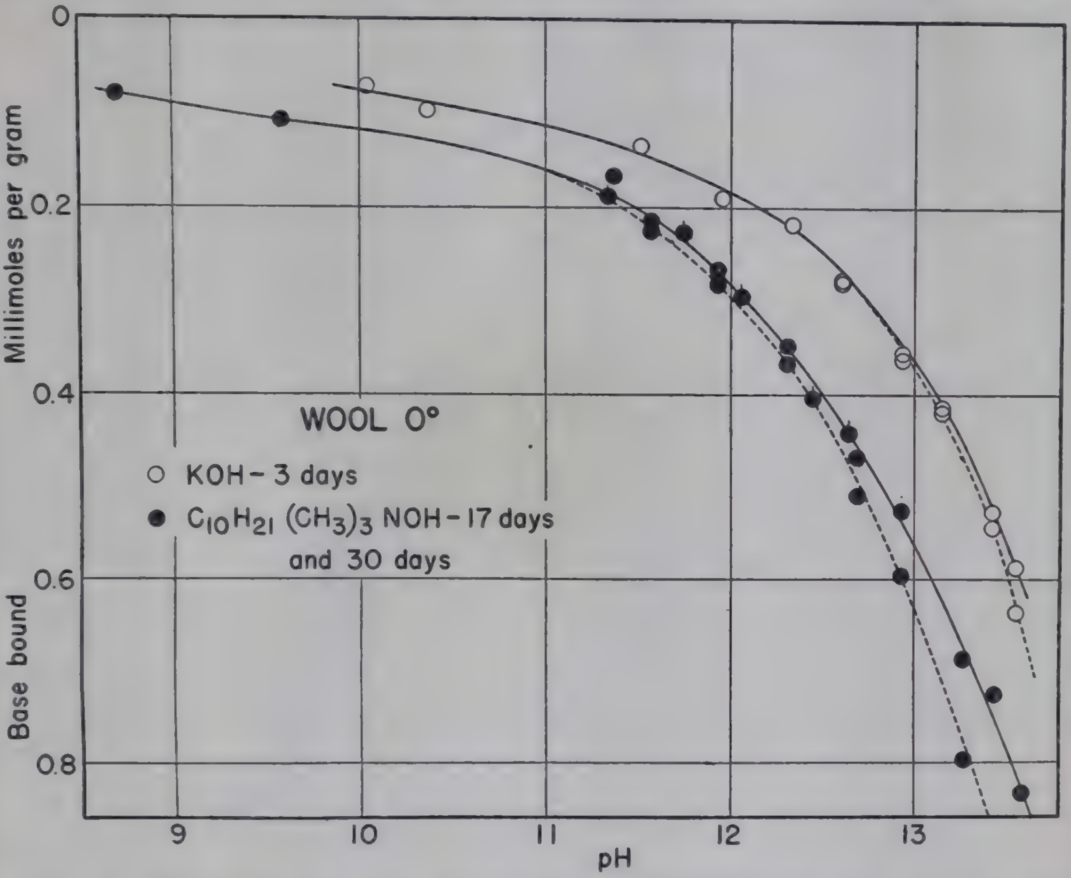

FIG. 2. Combination of wool protein with a representative pair of strong bases at 0°. The dotted lines represent uncorrected data, and the solid lines data corrected for the decomposition of disulfide bonds. The tagged circles represent measurements obtained upon reversal of the combination of protein with base. Note the large non-uniform shift in the curve for decyltrimethylammonium hydroxide.

sistent with the 17 day measurements. They demonstrate, therefore, that (*a*) the correction for decomposition is made correctly and (*b*) that the solid line represents the attainment of a truly reversible acid-base equilibrium.

The results obtained with six homologous compounds, alkyltrimethylammonium hydroxides, are shown together in Fig. 3. As the molecular

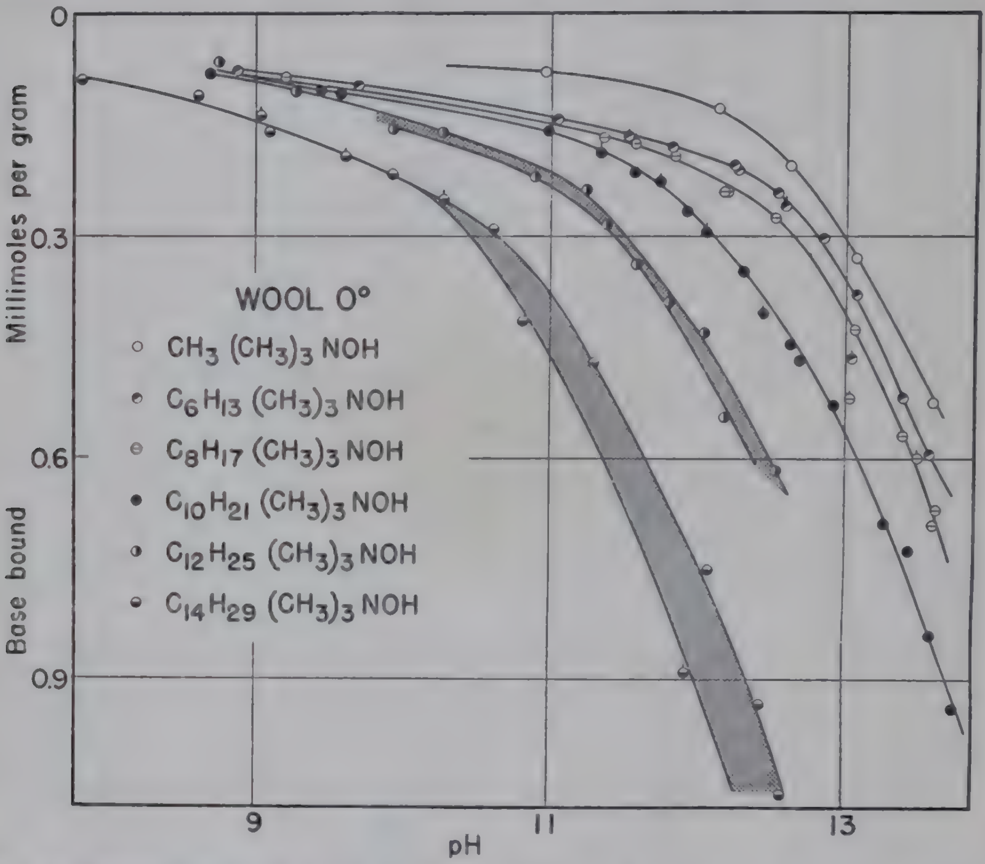

FIG. 3. Combination of wool protein with six homologous alkyltrimethylammonium hydroxides at 0°. The shaded areas represent the range of uncertainty in the equilibrium position of the curve, due to failure to attain final equilibrium in the long times (15 to 17 days) allowed for the two highest homologues. The tagged circles represent measurements obtained upon reversal of the combination of protein with base.

weight (and the length of the alkyl chain) increases, there is a progressive displacement of the titration curves to lower pH values; *i.e.*, more base is bound at lower base concentrations. The shift corresponding to each 2-carbon increment in chain length also becomes progressively larger. Thus, there is only a small difference between the results obtained with the lower homologues, but there is a very substantial difference between the decyl and dodecyl derivatives and an even larger difference between the dodecyl and tetradecyl. Similar shifts in acid titration curves have been shown to be the result of differences in the affinity of anions for the protein, and have been used to calculate these differences (14, 19). No such calculation is made here, because the complex nature of the titration curve in the alkaline region[3] would make the calculation somewhat arbitrary, but the qualitative conclusions are plain.

A striking feature of the data obtained with the decyl and tetradecyl derivatives is the large amount of base bound, 0.94 mM per gm. at pH 13.8 for the former compound and over 1.0 mM at pH 12.0 for the latter. Both figures exceed 0.8 mM per gm., accepted by earlier authors (12), even though the present curves give no indication of leveling off to a maximum value. On the basis of analytical data, the theoretical maximum base-binding capacity of wool is 1.18 mM per gm., if the content of tyrosine, 0.32 mM per gm., is added to the sum of the arginine and lysine, 0.86 mM per gm. (the latter is very nearly equal to the dicarboxylic acid content indicated by the acid titration curve). The assumption that the cationic form (RNH_3^+) of arginine is too weak an acid to be titrated in this range would reduce the maximum base bound to 0.59 mM per gm. This figure approximates the value found with the more common bases, in solutions containing about 0.05 to 0.07 molal free base. Thus, it appears that the electrostatic effect of combination of the protein with cations has weakened the proton affinity of arginine sufficiently to make it dissociate with low concentrations of base.

Similar effects must be produced on the cationic form of lysine, and on undissociated tyrosine. It will be shown below that the effect on lysine is much greater than the effect on tyrosine or arginine.

One cannot distinguish, within a single homologous series of cations, between differences in affinity due to their total mass, linear dimensions, or other spatial relationships, or to more specific chemical effects. In Fig. 4, some of the results already shown are compared with data for more symmetrical quaternary ions with molecular weight in the same range. The data obtained with tetraethylammonium hydroxide, which has only a slightly lower molecular mass than hexyltrimethylammonium hydroxide, show a significantly lower affinity for the symmetrical cations. Much more marked differences appear when tetrabutylammonium hydroxide is compared with an unsymmetrical molecule of approximately the same molecular weight, dodecyltrimethylammonium hydroxide. Here, there is a displacement to lower pH values of well over 1 pH unit (higher affinity) for the asymmetric cation, although the curves tend to come together for small amounts of base bound.

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[3] The curve of combination with acid can be characterized by a single pair of equilibrium constants, one for the dissociation of a single type of acid group (COOH), and one for the corresponding association with anions (14, 19). The base curve results from the dissociation of at least three proton donors (in lysine, arginine, and tyrosine) (1).

Data for a less asymmetric molecule, phenyltrimethylammonium hydroxide, are also shown in Fig. 4. The molecular weight of this compound is comparable with that of the more asymmetric hexyltrimethylammonium hydroxide, and the more symmetrical tetraethylammonium hydroxide. The results obtained are practically identical with the asymmetric ion at large amounts bound (shift in pH), but unlike the completely symmetric ions, the shift in pH is still great for small amounts bound. The reason for this marked specificity remains obscure.

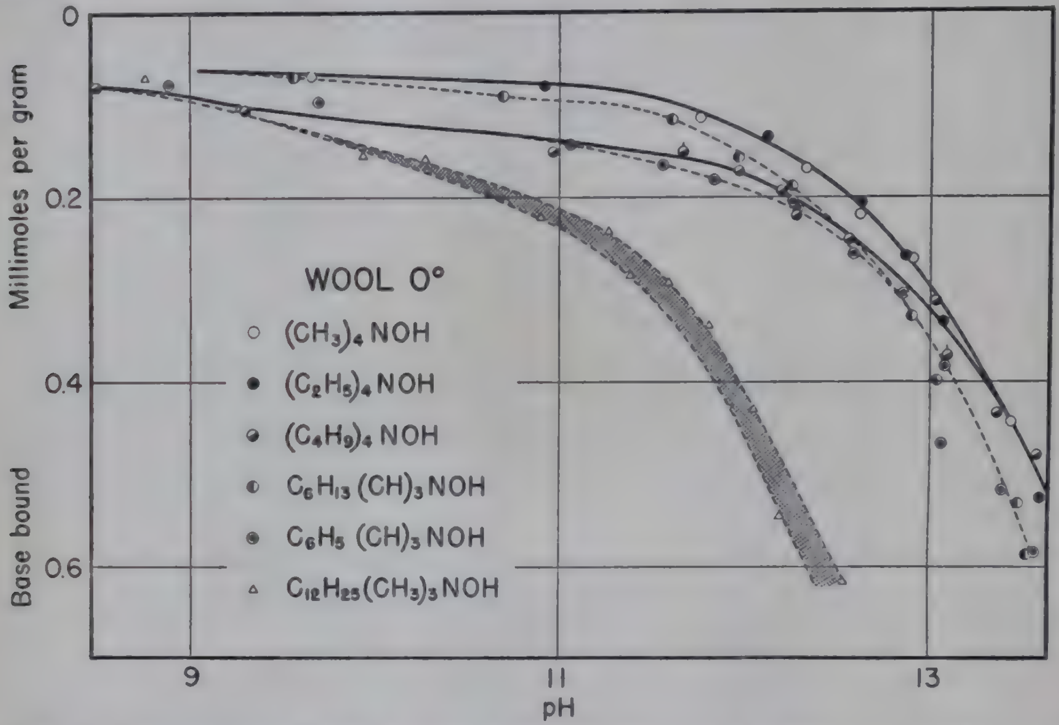

FIG. 4. Combination of wool protein with symmetric and asymmetric quaternary ammonium hydroxides at 0°. The tagged circles represent measurements obtained upon reversal of the combination of protein with base.

Experiments with univalent inorganic bases show only small differences (Fig. 5), although the spread in molecular weight is relatively large. All of the inorganic cations tested, however, had slightly higher affinities for wool than the organic cation of lowest molecular weight, tetramethylammonium.

Close examinations of the preceding figures will show that the shift in pH produced by the cations of higher affinity is not uniform along the entire curve, as it was in the case of titration with acid (19, 20); *i.e.*, the base curves are not exactly superimposable. The differences in displacement are made clear in Fig. 6 in which the displacements in pH for all bases (relative to the curve for tetramethylammonium hydroxide) are compared at various amounts bound. A large number of bases (predominantly those of low molecular weight) produce a relatively small displacement (<0.4 unit) over most of the titration curve, but all except the smallest of these produce a very much larger displacement when only small amounts (<0.2 mM per gm.) are bound. This larger shift occurs in the region of the titration curve corresponding to the dissociation of lysine. As the ion size increases, especially among the detergent (asymmetric) ions, much larger displacements are found all along the titration curve, but the lysine region continues to be displaced to a larger extent, about 1 pH unit more than the rest of the titration curve. The symmetrical tetrabutylammonium ion, however, behaves uniquely in this respect; it shows a maximum displacement of almost 2.5 pH units at about 0.1 mM bound, but no displacement at all at larger amounts (>0.4 mM per gm.) bound. Its selective effect on the lysine component is extremely sharp; in fact the shift in the lysine component which it produces is as great as the shift produced by the asymmetric ions of similar molecular weight, although its effect on the other components is negligible.[4]

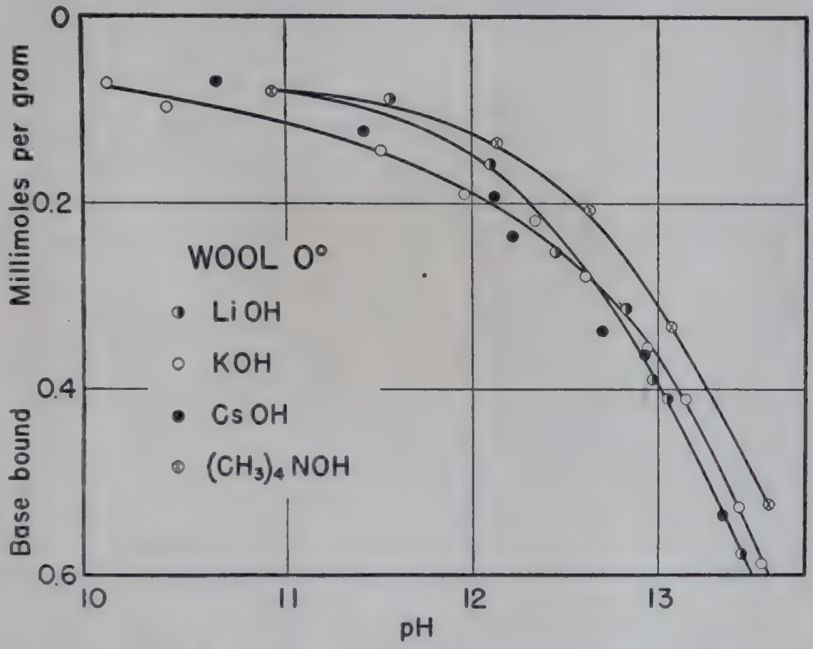

FIG. 5. Combination of wool protein with inorganic bases at 0°. The curve for the quaternary ammonium base of lowest molecular weight is inserted for comparison.

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[4] The fact that ions of relatively low detergency, phenyltrimethylammonium and tetrabutylammonium, are outstanding in the selectivity of their affinity effects in the lysine region tends to support the view that this effect is real, and not an artifact brought about by surface adsorption effects at the electrode.

The inclusion in Fig. 6 of data obtained with KOH in the presence of 1 M formaldehyde demonstrates the close parallel between the selective effect of large cations on the dissociation of lysine groups and the wholly similar effect of formaldehyde (22) which is known to be predominantly on lysine.

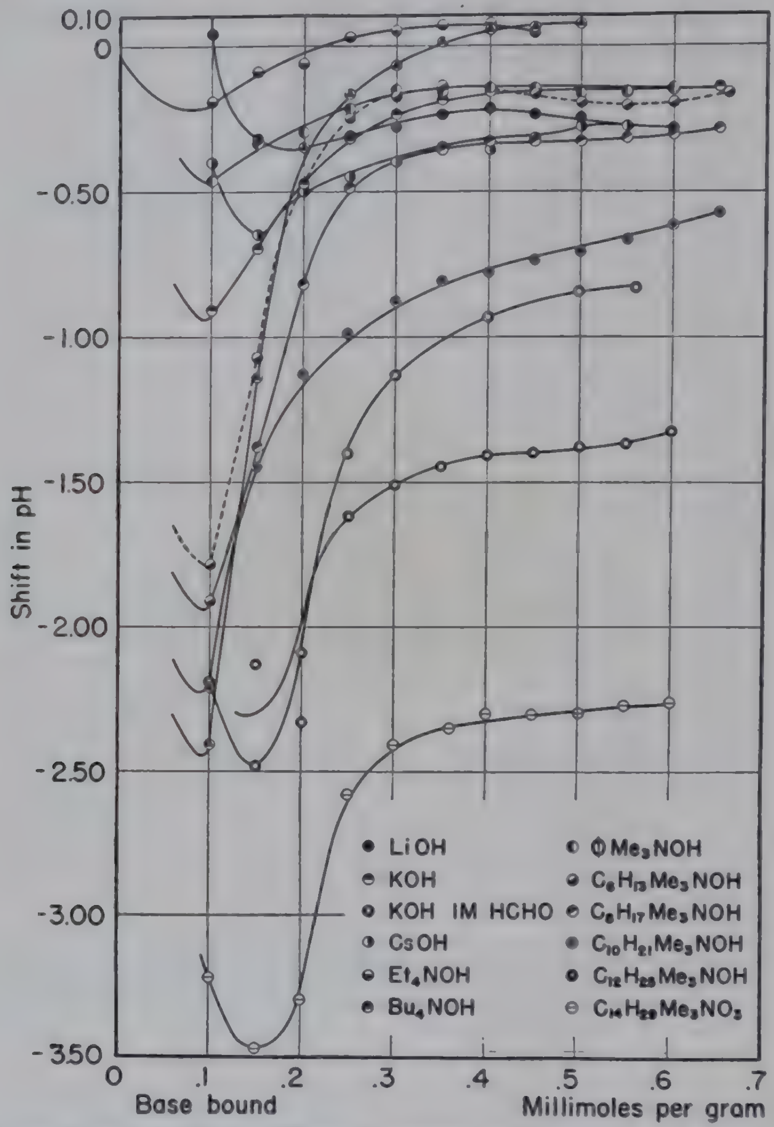

FIG. 6. Variation of shift in pH with amount of base bound for twelve bases. A curve showing the similar effect of formaldehyde is included for comparison. Displacements in pH are referred to the titration curve of tetramethylammonium hydroxide, which has the lowest affinity of any of the bases tested.

The selective effect of cations on the lysine component just described had no parallel in the earlier data obtained with acids. Although other causes for this effect are conceivable, it may merely indicate that the first cations which combine are bound more strongly than those which combine only when the base concentration is increased. If this is the case, the effect is not on lysine at all, but manifests itself on the lysine component of the titration curve only because the first considerable numbers of protons to dissociate, on addition of base, come from lysine ammonium groups, the strongest acidic groups remaining in the molecule. This explanation is consistent with other work (4, 9) which indicates that, with other proteins, a certain number of ions combine much more readily than the remainder. It is a necessary consequence of this explanation, however, that, with wool, all the anions which can combine are bound essentially equally tightly.

Forces Involved in Ion Binding by Protein

The results with anions reported earlier (13, 15, 19, 20) established that, although non-specific (*i.e.* van der Waals) forces, rather than primary chemical valences, were primarily responsible for the binding of anions by wool and egg albumin, the effect of these forces manifested itself only when long range (*i.e.* coulombic) forces increased the probability that the ions would approach closely enough for the van der Waals forces to operate (see (21) p. 422, foot-note). The principal evidence is summarized below, where it is compared with evidence from the present investigation.

Van der Waals Forces Involved—The affinity of anions has been shown to depend predominantly on molecular weight. The same relationship has been established in this paper for cations; and it has further been shown that molecular asymmetry can greatly enhance the affinity at a given molecular weight. Since this fact suggests that the *size* of the ion along its longest axis may be the determining factor, the data obtained with cations have been plotted against both molecular weight and length of major axis, as measured on fully extended Fischer-Hershfelder models. These comparisons are shown in the top and bottom portions respectively of Fig. 7. All of the data obtained with organic cations can be expressed as a smooth monotonic function of ion *size*, although they scatter widely (see especially tetrabutylammonium ion) when plotted against molecular weight. The inorganic ions, however, have higher affinities *per unit weight or size*, than do the organic ions; these ions, undoubtedly, present quite different surfaces to the protein; they alone are not covered with molecularly linked hydrogen.

Organic ions of small size (molecular weights below about 150 or sizes below about 12 to 14 A), differ very little in affinity among one another. Above these limits, the affinity increases rapidly, causing a displacement in pH, for a given amount bound, of about 1 unit for every increment in molecular weight of 45 (for asymmetric ions). Among the anions studied previously, the value at which a strong dependence on molecular weight begins is the same or slightly smaller, and the affinity increases about half as much per unit of molecular weight. The existence of minimum sizes for differences in affinity in both cases is similar to the conditions for micelle formation in compounds containing these ions.

Long Range Coulombic Forces Also Involved—This may be inferred from the fact that the combination with anions was stoichiometric and the maximum amount combined corresponded to the number of positively charged groups. When an apparent excess combination occurred (*i.e.* more than would be accounted for by back titration of COO⁻ groups), it could be shown that hydrogen ion also combined stoichiometrically with amide groups ($RCONH_3^+$) of glutamine and asparagine, preserving electrical neutrality. The fact that this slight excess combination was actually with the amide groups was shown by the experimental demonstration that hydrolysis of these groups always set in at the lowest concentrations of

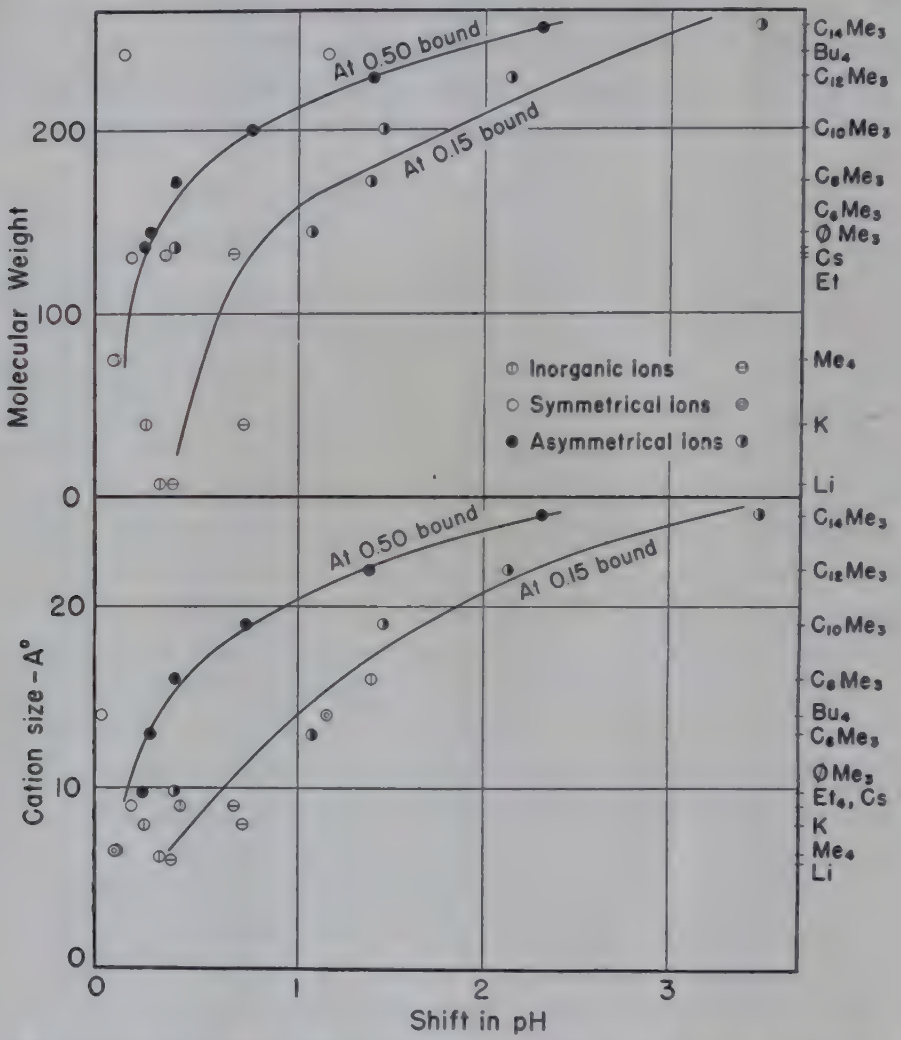

FIG. 7. Relation of shift in pH to molecular weight and length of cations. Curves are given for two different amounts of base bound to allow for the differences in effects produced at small and large amounts bound.

acid (different with each anion) at which the excess combination occurred (16).

In the experiments with base, stoichiometry cannot be demonstrated, since the maximum theoretical amounts of *base* were never bound within the range of concentrations employed. However, the fact that these maxima were only approached, and never exceeded, makes it unnecessary to assume that the cations combined, by van der Waals forces alone at uncharged sites, or by building up micellar layers on the first cations to combine at charged sites. Thus, it appears that, as with anions, the van der Waals forces alone are not sufficient to overcome the thermal energy dissociating these combinations, except in the vicinity of long range electrostatic forces which reinforce their effect.

Specific Chemical Bonds Not Involved—The best evidence for this thesis is the demonstration, in the work with anions, that the binding energy depended primarily on molecular size, with only secondary differences between non-homologous compounds. A much smaller range of chemical structures has been available with cations, but the results have been much the same. Furthermore, if specific chemical forces (sharing of electrons) are responsible at all, a complete electron pair (donated, for example, to the protein by the anion) would have to be involved. The formation of such a dative bond in the vicinity of the RNH_3^+ groups of the protein is impossible because all the atoms of these groups already have complete valence shells. Although the *anions* might donate an electron pair elsewhere in the protein molecule, the organic cations used in this study could neither donate nor accept pairs. Since cations and anions of the same size have very similar affinities for the protein, the formation of covalent bonds (other than hydrogen bonds) cannot be involved.

Thanks are due to Professor George Scatchard of the Department of Chemistry, Massachusetts Institute of Technology, for extension of the Department's facilities, and for many helpful discussions.

SUMMARY

The titration curves of wool at 0° with twelve strong monovalent bases exhibit similar wide differences with respect to position on the pH axis, as has been previously demonstrated with acids. Thus, as with anions, the affinity of cations for protein increases with increasing molecular dimensions. Among organic cations of the same weight, asymmetric ions produce much the larger shift. Unlike the effects of anions, which are uniform over the entire acid curve, the effect of cations is more marked on the portion of the titration curve which is also affected by formaldehyde; *i.e.*, the part due to lysine. The maximum shift in position of this part of the titration curve, obtained with tetradecyltrimethylammonium hydrox-

ide, exceeds 2 pH units. Marked effects on other portions of the curve are shown only by asymmetric ions larger than the hexyltrimethylammonium ion. Explanations of this distinction have been suggested, as well as of the selective effect on the dissociation of lysine. As with anions, the binding of cations is brought about by van der Waals forces which have an opportunity to manifest themselves only when long range (electrostatic) forces increase the probability of close enough approach. No specific chemical bonds are involved.

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